# BIOGENESIS OF RAT LIVER PEROXISOMES: CHARACTERIZATION OF THE INTEGRAL MEMBRANE POLYPEPTIDES AND IDENTIFICATION OF A NOVEL 3-KETOACYL-CoA THIOLASE

By

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# BIOGENESIS OF RAT LIVER PEROXISOMES

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TITLE: Biogenesis of Rat Liver Peroxisomes: Characterization of the Integral Membrane Polypeptides and Identification of a Novel 3-Ketoacyl-CoA Thiolase

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## ABSTRACT

This thesis is divided into two parts, each part addressing a particular aspect of the biogenesis of rat liver peroxisomes. The first part describes the characterization of the peroxisomal integral membrane polypeptides, and the second part describes the primary structure and targeting of peroxisomal 3-ketoacyl-CoA thiolase.

In the first part, membranes were isolated from liver peroxisomes of untreated rats and rats treated with clofibrate, a hypolipidemic drug which induces peroxisomal proliferation in rodent hepatocytes. The membranes were used to raise an antiserum in rabbits that reacted with six peroxisomal integral membrane polypeptides (molecular masses 140, 69, 50, 36, 22 and 15 kDa). Treatment of rats with clofibrate caused a 4- to 10-fold induction in the 69 kDa integral membrane polypeptide, while the other integral membrane polypeptides remained unchanged or varied to a lesser extent. The anti-peroxisomal membrane serum reacted with two integral membrane polypeptides of the endoplasmic reticulum which co-migrated with the 50 kDa and 36 kDa integral membrane polypeptides of peroxisomes. Subcellular fractionations and immunoblot analyses indicated that these integral membrane polypeptides were co-localized to peroxisomes and endoplasmic reticulum. Immunoprecipitation of *in vitro* translation

products of RNA isolated from free and membrane-bound polysomes indicated that the 22, 36, and 69 kDa integral membrane polypeptides were synthesized on free polysomes, while the 50 kDa integral membrane polypeptide was predominantly synthesized on membrane-bound polysomes. The predominant synthesis of the 50 kDa integral membrane polypeptide on membrane-bound polysomes raises interesting possibilities concerning its biosynthesis and trafficking.

3-Ketoacyl-CoA thiolase (thiolase) catalyzes the final step of the fatty acid ß-oxidation pathway in peroxisomes. Thiolase is unique among rat liver peroxisomal enzymes in that it is synthesized as a precursor possessing an amino-terminal extension, which is cleaved to generate the mature enzyme. To facilitate further examination of the synthesis, intracellular transport and processing of this enzyme, cDNA recombinants encoding thiolase were selected from a $\lambda$gt11 rat liver library using antiserum raised against peroxisomal thiolase. Upon sequencing several cDNA recombinants, it was revealed that there are two distinct thiolase enzymes in rat liver peroxisomes, thiolase 1 and thiolase 2. The cDNAs encoding thiolases 1 and 2 exhibited 94.6% nucleotide sequence identity and 95.4% identity at the level of deduced amino acid sequence. Interestingly, the expression of the genes encoding the two thiolases was differentially regulated. The mRNA encoding thiolase 1 was expressed at a low level in untreated

rat liver and was induced greater than 10-fold upon treatment with clofibrate. In contrast, the mRNA encoding thiolase 2 appeared to be constitutively expressed in rat liver and was only slightly induced upon treatment of rats with clofibrate. The cDNA encoding peroxisomal thiolase 2 was used in *in vivo* targeting studies which indicated that the thiolase prepiece contains information for targeting this protein to peroxisomes.

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| | |
|---|---|
| A | Ampere |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| bp | base pair |
| BSA | bovine serum albumin |
| CAT | chloramphenicol acetyltransferase |
| cDNA | DNA complementary to RNA |
| Ci | Curie(s) |
| CoA | coenzyme A |
| cpm | counts per minute |
| Da | dalton |
| ddNTP | 2',3'-dideoxynucleoside 5'-triphosphate |
| DHAP | dihydroxyacetone phosphate |
| DHCA | 3α,7α-dihydroxy-5β-cholestanoic acid |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside 5'-triphosphate |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| ER | endoplasmic reticulum |
| g | gram(s) |
| $g_{av}$ | acceleration of gravity (average) |

| | |
|---|---|
| $g_{max}$ | acceleration of gravity (maximum) |
| G-3-P | glycerol-3-phosphate |
| GTP | guanosine 5'-triphosphate |
| HEPES | n-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) |
| HSP | heat shock protein |
| IgG | immunoglobulin G |
| IgG(Fc) | constant region of immunoglobulin G |
| IMP | integral membrane polypeptide |
| IPTG | isopropyl ß-D-thiogalactoside |
| k | kilo |
| $\mu$ | micro |
| m | milli |
| M | molar |
| min | minute(s) |
| mol | mole(s) |
| $M_r$ | relative molecular mass |
| mRNA | messenger ribonucleic acid |
| n | nano |
| NAD | nicotinamide adenine dinucleotide (oxidized form) |
| NADH | nicotinamide adenine dinucleotide (reduced form) |
| NADP | nicotinamide adenine dinucleotide phosphate (oxidized form) |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced form) |
| NBT | nitroblue tetrazolium |
| NP-40 | Nonidet P-40 |

| | |
|---|---|
| NTP | nucleoside 5′-triphosphate |
| $OD_x$ | optical density measured at the wavelength of x nanometers |
| ORF | open reading frame |
| p | pico |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| pfu | plaque forming unit(s) |
| PNS | post-nuclear supernatant |
| PPO | 2,5-diphenyloxazole |
| PTS | peroxisomal targeting signal |
| RACE | rapid amplification of cDNA ends |
| rER | rough endoplasmic reticulum |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| sER | smooth endoplasmic reticulum |
| SM | simplified medium |
| SRP | signal recognition particle |
| SSC | sodium chloride-sodium citrate buffer |
| TBE | Tris-borate-EDTA buffer |
| TBST | Tris-buffered saline plus Tween 20 |

| | |
|---|---|
| TCA | trichloroacetic acid |
| TE | 10 mM Tris-HCl (pH 7.5), 1mM EDTA |
| TEMED | N,N,N′,N′-tetramethylethylenediamine |
| THCA | 3α,7α,12α-trihydroxy-5β-cholestanoic acid |
| Tris | Tris(hydroxymethyl)aminoethane |
| tRNA | transfer ribonucleic acid |
| Tween 20 | polyoxyethylenesorbitan monolaurate |
| V | volt(s) |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactoside |

# INTRODUCTION

Peroxisomes of animal tissues were once considered to be fossil organelles (de Duve, 1970). However, the abundance of peroxisomes in certain tissues (*e.g.* liver, kidney), their high turnover rate and their ability to proliferate in response to various drugs argued for an active role in metabolism (de Duve, 1970). It is now known that mammalian peroxisomes perform many diverse functions essential to cellular metabolism and proper development.

Since their discovery, uncertainty has also surrounded the biogenesis and origin of peroxisomes. This controversy has only recently been addressed with the development of modern experimental techniques, including the ability to isolate highly purified organelles. These advances have contributed greatly to our current understanding of the properties, functions and biogenesis of mammalian peroxisomes.

## 1.1 General Characteristics of Peroxisomes

The peroxisome is a cellular organelle (0.5 - 1.5 $\mu$m in diameter) consisting of a single unit membrane, a granular matrix and often a paracrystalline core (Tolbert, 1981). They are often spherical, but they may also form small tubules and interconnected networks referred to as the "peroxisomal

reticulum" (Lazarow and Fujiki, 1985). Peroxisomes are present in virtually all eukaryotic cells, with the exception of erythrocytes, and their abundance varies from one to hundreds per cell (Tolbert, 1981; Lazarow and Fujiki, 1985). In mammalian tissues, they are most abundant in the liver, kidney and brain (Tolbert, 1981).

Mammalian peroxisomes were first detected by J.A. Rhodin in 1954 while studying micrographs of mouse kidney cells (de Duve and Baudhuin, 1966). Rhodin reported a small organelle consisting of a single membrane with a fine, granular matrix, which he termed the "microbody". Later, Rouiller and Bernhard (1956) observed similar structures in rat liver; however, in this tissue they contain a dense paracrystalline core structure.

Mammalian peroxisomes were characterized further by the pioneering subcellular fractionation experiments of de Duve and his colleagues (de Duve and Baudhuin, 1966). Subcellular fractionation of rat liver established that microbodies are distinct from lysosomes and mitochondria and contain oxidases (*e.g.* urate oxidase and D-amino acid oxidase), which form $H_2O_2$, and catalase which reduces $H_2O_2$ (de Duve and Baudhuin, 1966). As a consequence of its inherent $H_2O_2$ metabolism, de Duve proposed that the microbody be renamed the peroxisome (de Duve and Baudhuin, 1966). Since that time, a variety of oxidative and other enzymatic functions have been localized to peroxisomes. However, the enzyme complement of

this organelle can vary depending on cell type, available substrate, stage of development and exposure to chemical agents (Tolbert, 1981). Because the enzyme complement within peroxisomes of different species can vary, the term "microbody" has been retained and has come to describe, in general terms, this heterogeneous group of organelles. This group of organelles includes the peroxisome, found in most eukaryotic cells, the glyoxysome of plants and the glycosome of *Kinetoplastida* (Borst, 1986; 1989). These organelles contain a diversity of enzyme systems; however, they have in common their morphology and some enzymes. In addition to a ß-oxidation pathway, glyoxysomes of plant seeds contain the enzymes of the glyoxylate cycle (Beevers, 1982). The glycosomes of *Kinetoplastida* contain a large part of the glycolytic pathway and share with peroxisomes the first enzymes in the pathway of ether lipid biosynthesis (Hajra and Bishop, 1982; Opperdoes, 1984). The term microbody will be used on occasion throughout this thesis to refer to the more general family of organelles including the peroxisomes, glyoxysomes and glycosomes.

## 1.2 Functions of Mammalian Peroxisomes

The enzyme complement of peroxisomes varies depending on the cell type and growth conditions (Tolbert, 1981). This is not only true for microbodies of different species but also for microbodies of a single species in which there is often

heterogeneity of microbodies from different tissues (Tolbert, 1981). This heterogeneity indicates that microbodies will eventually have to be characterized from each tissue for a complete list of their functions (Tolbert, 1981). Unless otherwise stated, this discussion will concentrate on mammalian hepatic peroxisomes, which is one of the most extensively studied systems.

Mammalian peroxisomes perform many diverse functions including respiration and metabolism of lipids, carbohydrates, amino acids and purines (Mannaerts and van Veldhoven, 1990). Peroxisomes were initially characterized based on their respiratory functions. They contain a variety of oxidases that act on lactate, glycolate, urate, polyamines, amino acids, fatty acyl-CoAs and other compounds generating $H_2O_2$ (Lazarow, 1987). Subsequently the $H_2O_2$ is decomposed by catalase. Unlike respiration in other cellular locations, respiratory reactions in the peroxisome do not conserve the energy of oxidation as ATP but are thermogenic (Lazarow, 1987).

More than half the enzymes of mammalian peroxisomes are involved in lipid metabolism (Mannaerts and van Veldhoven, 1990). Peroxisomes play a role in the ß-oxidation of fatty acids (Lazarow and de Duve, 1976), cholesterol biosynthesis (Appelkvist *et al*, 1990), the formation of bile acids (Pedersen and Gustafsson, 1980) and plasmalogen biosynthesis (Hajra *et al*, 1979). These functions, as well as

carbohydrate, amino acid and purine metabolism will be discussed in the following section.

### 1.2.1 ß-oxidation Pathway

The peroxisomal ß-oxidation pathway has been identified in a wide variety of cell types (*Tetrahymena*, mammals, yeast and germinating castor bean endosperm). Therefore ß-oxidation is an important general function of microbodies (Tolbert, 1981).

Until Lazarow and de Duve (1976) reported the existence of a ß-oxidation system in rat liver peroxisomes, ß-oxidation of fatty acids was believed to occur solely in the mitochondrial matrix of mammalian cells. Since that time many studies on the significance of the peroxisomal system have been undertaken. The sequence of reactions of the fatty acid degradation pathway in peroxisomes is summarized in Fig. 1.2.1. The reaction sequence is similar to that carried out by mitochondria; however, the proteins involved have distinct molecular and catalytic properties, and the specificity and regulation of the two pathways are different (Tolbert, 1981; Hashimoto, 1982; Lazarow and Fujiki, 1985).

In the initial step, fatty acid is activated to fatty acyl-CoA by an ATP-dependent acyl-CoA synthase (Hashimoto, 1982). Subsequently, fatty acyl-CoA is reduced to trans-2-enoyl-CoA with the consumption of oxygen and the production of $H_2O_2$, which in turn is decomposed by catalase (Tolbert, 1981;

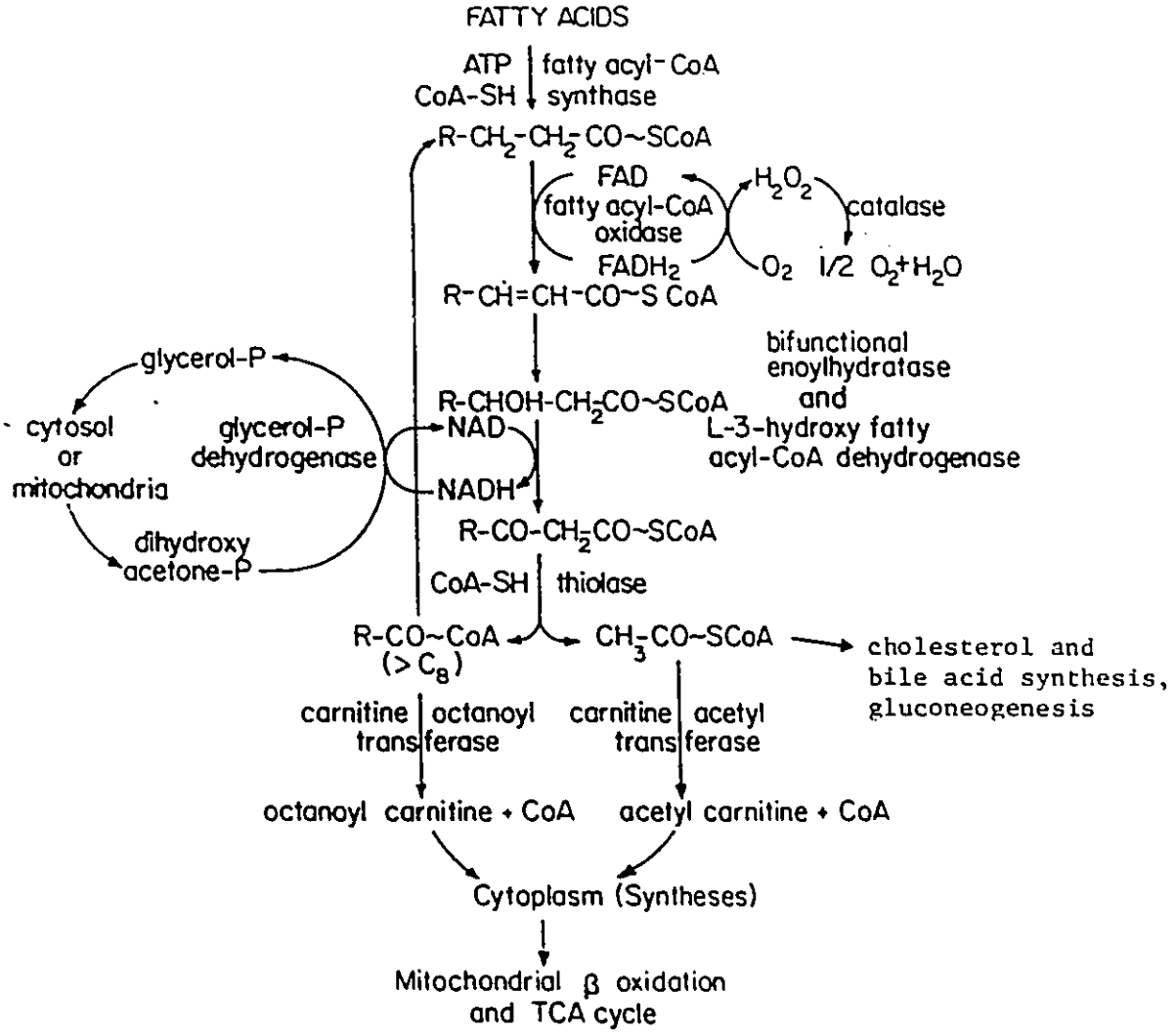



Fig. 1.2.1. Fatty acid ß-oxidation pathway in liver peroxisomes. Adapted from Tolbert (1981).

Hashimoto, 1982). This reaction is catalyzed by acyl-CoA oxidase, which is rate-limiting in peroxisomes (Hashimoto, 1982). The following two reactions, which are catalyzed by two independent enzymes in mitochondria, are catalyzed by a multifunctional peroxisomal enzyme exhibiting both enoyl-CoA hydratase and hydroxyacyl-CoA dehydrogenase activities (hereafter referred to as hydratase dehydrogenase) (Tolbert, 1981; Hashimoto, 1982; Lazarow and Fujiki, 1985). In the final reaction, 3-ketoacyl-CoA is cleaved by 3-ketoacyl-CoA thiolase into acetyl-CoA and an acyl-CoA having two carbons less than the starting molecule (Hashimoto, 1982). The acyl-CoA ($>C_8$) can re-enter the pathway, whereas the acetyl-CoA and acyl-CoA ($\leq C_8$) are transported to the mitochondria or used as substrates in peroxisomal anabolic processes (Tolbert, 1981; Hashimoto, 1982). The peroxisomal matrix contains soluble carnitine acetyltransferase and carnitine octanoyltransferase that can convert products of peroxisomal ß-oxidation to carnitine esters, which can be oxidized further in the mitochondria (Markwell *et al*, 1976; Tolbert, 1981; Mannaerts and Debeer, 1982). In addition to supplying acetyl-CoA and acyl-CoA for mitochondrial energy production, peroxisomal acetyl-CoA serves as a substrate for cholesterol and bile acid biosyntheses and gluconeogenesis (Tolbert, 1981; Hayashi and Takahata, 1991).

The peroxisomal and mitochondrial ß-oxidation pathways exhibit different fatty acid chain-length specificities.

Mitochondria readily oxidize short-, medium- and long-chain fatty acids (up to $C_{18}$), while peroxisomes efficiently oxidize medium-, long- and very long-chain fatty acids ($C_{10}$ to $C_{30}$) and show little activity towards fatty acids shorter than octanoic acid (Tolbert, 1981; Mannaerts and Debeer, 1982).

In addition to medium-, long- and very long-chain fatty acids, peroxisomal ß-oxidation can utilize a number of other substrates which are not efficiently oxidized by mitochondria. These substrates include: unsaturated fatty acids (Osmundsen, 1982; Osmundsen and Hovik, 1988), medium- and long-chain dicarboxylic acids (van Hoof *et al*, 1988), the carboxy-side chains of prostaglandins (Diczfalusy and Alexson, 1990), trihydroxycholestanoic acid (an intermediate of bile acid formation) (Pedersen and Gustafsson, 1980), cholesterol (Hagey and Krisans, 1982) and xenobiotic fatty acids (Yamada *et al*, 1986).

Unlike the mitochondrial pathway, the peroxisomal ß-oxidation pathway is not coupled to the electron transport chain and is therefore insensitive to cyanide and other inhibitors of mitochondrial electron transport (Hashimoto, 1982; Mannaerts and Debeer, 1982). In peroxisomes, energy released at the second dehydrogenase step is partly conserved as NADH, which can be shuttled to the cytosol and mitochondria, whereas the energy of the first oxidation step catalyzed by acyl-CoA oxidase is lost as heat (Mannaerts and Debeer, 1982).

Interestingly, the peroxisomal ß-oxidation capacity is increased several-fold by treatment with a number of chemicals or by a high-fat diet (Lazarow and de Duve, 1976; Mannaerts and Debeer, 1982; Osmundsen, 1982). Treatment of rodents with several hypolipidemic drugs and organic plasticizers causes a marked proliferation of liver peroxisomes and a concomitant increase in ß-oxidation activity (see Introduction, section 1.5).

### 1.2.2 Cholesterol Biosynthesis

The pathway of cholesterol synthesis involving cytosolic and microsomal enzymes is well characterized (Fig. 1.2.2). The conversion of acetyl-CoA to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) occurs in the cytoplasm. The reduction of HMG-CoA to mevalonate is catalyzed by HMG-CoA reductase, the key regulatory enzyme of the sterol biosynthetic pathway located in the ER membrane. Subsequently, mevalonate is phosphorylated and decarboxylated to isopentenyl pyrophosphate. These reactions are followed by the synthesis of geranyl pyrophosphate, farnesyl pyrophosphate and squalene, which cyclizes to lanosterol. In the final reactions three methyl groups are removed from lanosterol, and the migration and reduction of double bonds produces cholesterol via nineteen discrete reactions (Thompson *et al*, 1987). Except for HMG-CoA reductase, the enzymes which convert acetyl-CoA to farnesyl pyrophosphate (a common intermediate for the

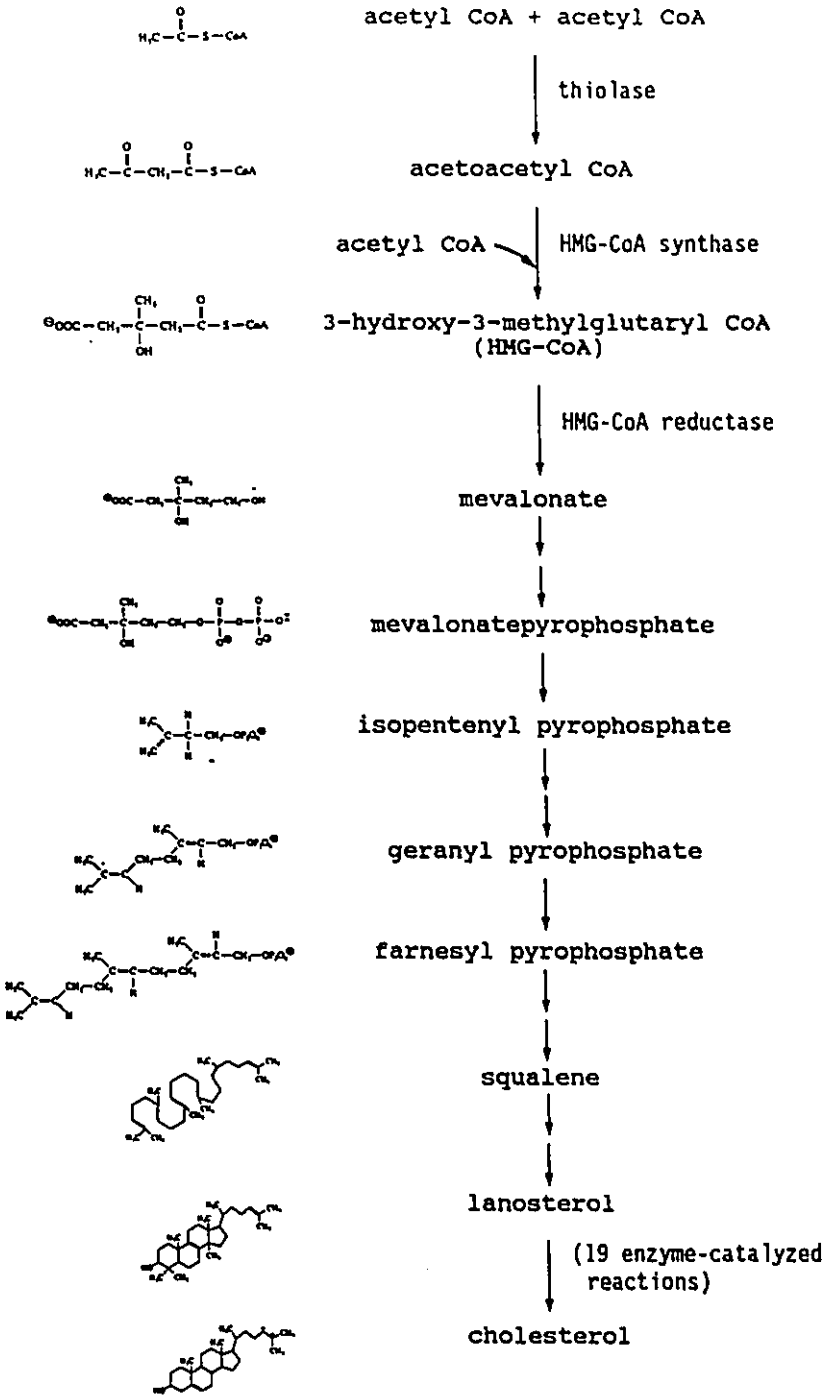



Fig. 1.2.2. The cholesterol biosynthetic pathway in mammals. Adapted from Rawn (1989).

biosynthesis of cholesterol, ubiquinone and dolichol) are found in the cytoplasm (Applekvist, 1990). The enzymes responsible for the conversion of farnesyl pyrophosphate to cholesterol are microsomal (Thompson *et al*, 1987).

Recently, peroxisomes have been found to play a role in cholesterol biosynthesis. The key regulatory enzyme HMG-CoA reductase, a transmembrane glycoprotein of the ER, has also been found in the matrix of rat liver peroxisomes (Keller *et al*, 1985). There is also evidence that rat liver peroxisomes contain the thiolase activity necessary for the initial step in cholesterol synthesis, the condensation of acetyl-CoA into acetoacetyl-CoA (Thompson and Krisans, 1990). In addition, it has been shown that purified peroxisomes can convert mevalonic acid to cholesterol in the presence of cytosolic proteins (Thompson *et al*, 1987; Appelvist *et al*, 1990). Further, sterol carrier protein 2 (SCP-2), which has non-enzymatic functions in cholesterol biosynthesis and utilization, has been recently localized to rat liver peroxisomes (Keller *et al*, 1989). This protein performs a variety of carrier functions *in vitro* associated with cholesterol biochemistry but also has a stimulatory effect on enzymes involved in cholesterol biosynthesis and utilization (Keller *et al*, 1989).

The contribution of the peroxisomal cholesterol biosynthetic pathway to overall cholesterol homeostasis has not been determined. Why certain steps of the cholesterol

synthetic pathway occur in two distinct intracellular locations is also not known. This may create two cellular sites of regulation of cholesterol biosynthesis or may allow the cell to channel newly synthesized cholesterol to distinct metabolic fates (Keller *et al*, 1985). Whether the individual steps of the pathways and the enzymes involved are identical in both locations remains to be determined.

### 1.2.3 Formation of Bile Acids

The major degradation pathway of cholesterol in mammals is the production of bile acids. The primary bile acids in mammals (cholic and chenodeoxycholic acid) are produced from cholesterol by a series of hydroxylation and oxidation reactions in the liver and are secreted with bile into the intestine as conjugates of taurine or glycine (Björkhem, 1985).

Until recently, the conversion of cholesterol and its derivatives to bile acids was thought to involve enzymes of the ER, mitochondria and the soluble components of the cell (Pedersen and Gustafsson, 1980). Pedersen and Gustafsson (1980) showed that peroxisomes are also involved in the formation of bile acids. The major pathway for bile acid synthesis begins in the ER where alterations of the steroid nucleus by ER and soluble enzymes are followed by the hydroxylation of the $C_{26}$ by either ER or mitochondrial hydroxylases and then by side-chain oxidation in the

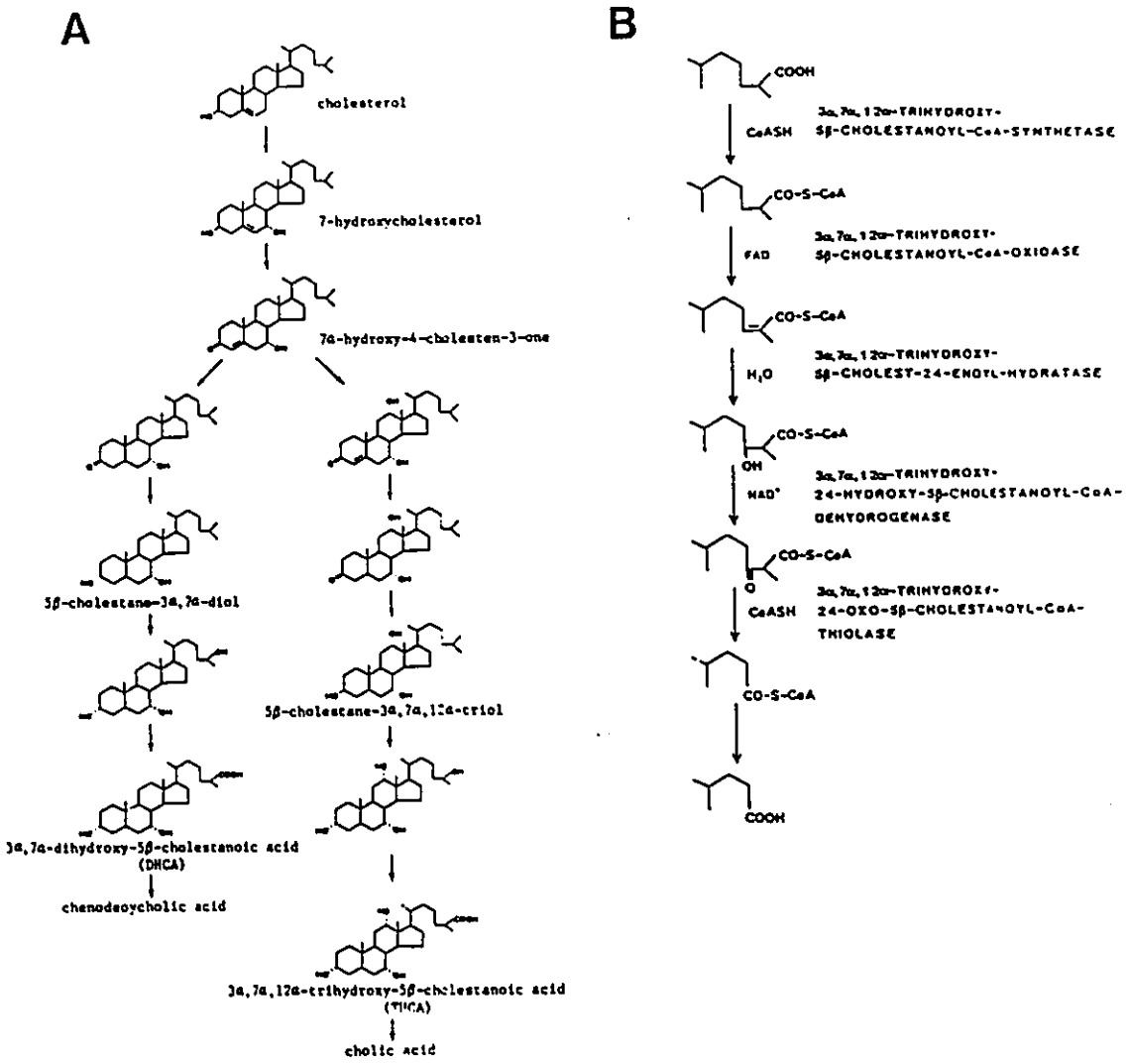



Fig. 1.2.3. Major reaction pathway for the biosynthesis of cholic acid and chenodeoxycholic acid from cholesterol. A: reactions from cholesterol to THCA and DHCA (adapted from Björkhem, 1985 and Björkhem *et al*, 1985). B: Proposed reaction pathway for side-chain cleavage in the conversion of THCA and DHCA to primary bile acids (Farrants *et al*, 1989).

mitochondria to produce a C-24 bile acid (Fig. 1.2.3, Björkhem, 1985). The reaction pathway that performs the oxidative cleavage of the C-27-steroid side-chain converting 3α,7α,12α-trihydroxy-5ß-cholestanoic acid (THCA) to cholic acid and 3α,7α,-dihydroxy-5ß-cholestanoic acid (DHCA) to chenodeoxycholic acid has been localized to the peroxisomal fraction of rat liver (Pedersen and Gustafsson, 1980; Kase *et al*, 1983; Pedersen *et al*, 1987). The oxidation pathway is thought to involve reactions similar to those of peroxisomal fatty acid ß-oxidation (Fig. 1.2.3, panel B; Farrants *et al*, 1989), but it is not known whether the same peroxisomal ß-oxidation enzymes are responsible for both pathways (Kase *et al*, 1983; Farrants *et al*, 1989).

The final thiolytic cleavage in the oxidation of the steroid side-chain is followed by conjugation of the bile acid with taurine or glycine (Björkhem, 1985; Pedersen *et al*, 1987). Recently, bile acid-CoA:amino-acid N-acetyltransferase, the enzyme responsible for this reaction, has been localized to rat liver peroxisomes (Kase and Björkhem, 1989). Therefore it appears that peroxisomes are capable of oxidative cleavage of the C-27-steroid side-chain as well as conjugation of the primary bile acids to glycine or taurine prior to their release into the circulation.

A minor alternative pathway for bile acid synthesis involves $C_{26}$ hydroxylation and side-chain oxidation of cholesterol prior to steroid hydroxylation (Fig. 1.2.4).

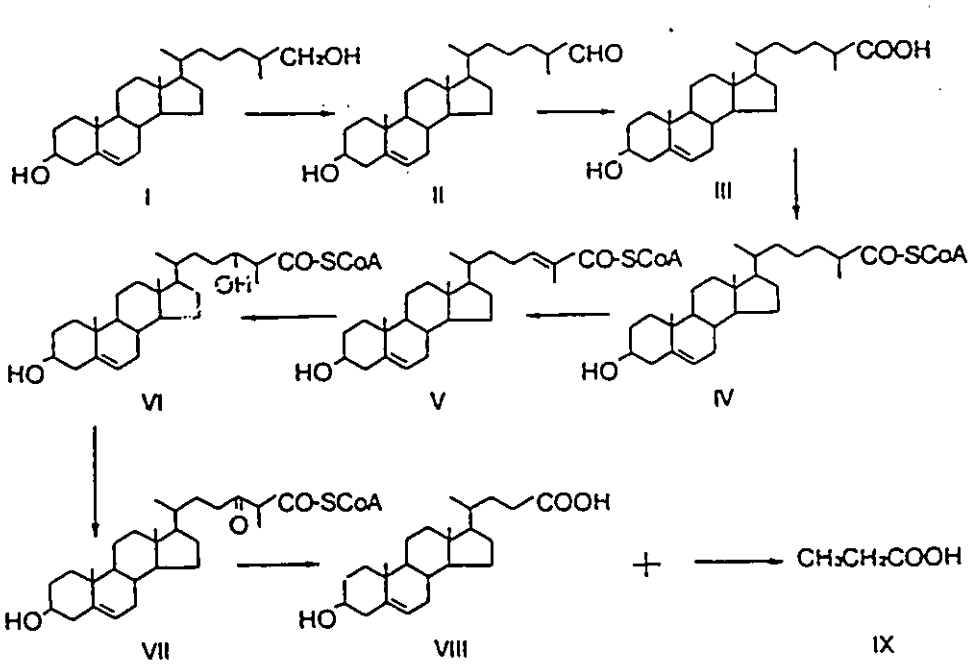



Fig. 1.2.4. The proposed peroxisomal reaction pathway for the side-chain modification of 26-hydroxycholesterol to 3ß-hydroxy-5-cholenoic acid. I, 26-hydroxycholesterol; II, 3ß-hydroxy-5-cholestenal; III, 3ß-hydroxy-5-cholestenoic acid; IV, 3ß-hydroxy-5-cholestenoyl CoA; V, 3ß-hydroxy-24-ene-5-cholestenoyl CoA; VI; 3ß-24-dihydroxy-5-cholestenoyl CoA; VII, 3ß-hydroxy-24-keto-5-cholestenoyl CoA; VIII, 3ß-hydroxy-5-cholenoic acid, IX, propionic acid. Reproduced from Krisans *et al* (1985).

Peroxisomes are also thought to contribute to this pathway as they have the capacity to oxidize 26-hydroxycholesterol (the product of the 26-hydroxylation of cholesterol) to 3ß-hydroxy-5-cholestenoic acid (Krisans *et al*, 1985).

The physiological significance of the peroxisomal contribution to bile acid formation is unknown (Pedersen and Gustafsson, 1980). However, it is thought to be significant based on results from studies of patients with Zellweger Syndrome, a disease characterized by the absence of functional peroxisomes. There is an accumulation of THCA and other bile acid intermediates with uncleaved steroid side-chains in the bile and plasma of these patients (Kase *et al*, 1985a; 1985b; Pedersen *et al*, 1987). This is probably due to defective peroxisomal oxidation of the side-chains of THCA and DHCA. The pool size and rate of formation of cholic acid are reduced to approximately one-tenth of normal in these patients (Pedersen *et al*, 1987).

### 1.2.4 Synthesis of Ether-Linked Glycerolipids

Ether-linked glycerolipids have been found in all mammalian cells (Zoeller *et al*, 1988). Plasmalogens, a class of unsaturated ether phospholipids, constitute a substantial fraction of the phospholipid of heart and brain and ether-linked glycerolipids are constituents of platelet activating factor (Snyder, 1972; Zoeller *et al*, 1988).

The biosynthesis of ester- and ether-linked

glycerolipids occurs largely in the ER; however, peroxisomes are responsible for catalyzing the first reactions in ether-linked glycerolipid synthesis (Hajra and Bishop, 1982). Glycerolipid synthesis starts with the acylation of either glycerol-3-phosphate (G-3-P) or dihydroxyacetone phosphate (DHAP) (Fig. 1.2.5). Acyl-DHAP has been shown to be a precursor of glycerolipids containing ester and ether bonds (Hajra and Bishop, 1982). It can enter the G-3-P pathway, which is responsible for synthesizing most glycerolipids, or it can enter the DHAP pathway, which produces ether-linked glycerolipids, including plasmalogens (Fig. 1.2.5) (Hajra and Bishop, 1982). The first three enzymes of the DHAP pathway have been localized to peroxisomes. Hajra *et al* (1979) demonstrated that rat liver and brain peroxisomes contain DHAP acyltransferase and acyl-DHAP:NADPH oxidoreductase. Later, it was shown that liver and brain peroxisomes also contain alkyl-DHAP synthase (Hajra and Bishop, 1982; Hardeman and van den Bosch, 1988). While DHAP acyltransferase and alkyl-DHAP synthase appear to be localized exclusively to the inner surface of the peroxisomal membrane, acyl-DHAP:NADPH oxidoreductase is distributed to both the peroxisomal and microsomal fractions (Hajra and Bishop, 1982). The subsequent reactions of ether glycerolipid synthesis occur in the ER (Hajra and Bishop, 1982).

The reason for the peroxisomal localization of some of the enzymes of the DHAP pathway is not clear. The apparent

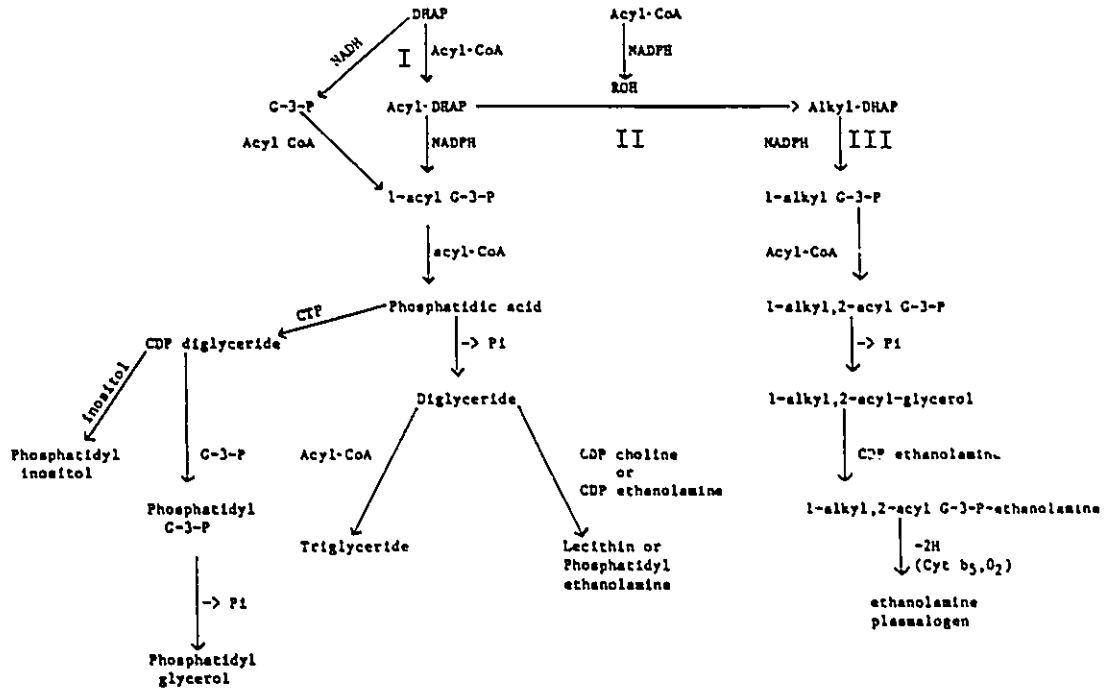



Fig. 1.2.5. The pathways for glycerolipid biosynthesis. The enzymes DHAP acyltransferase (I), alkyl-DHAP synthetase (II) and acyl-dihydroxyacetonephosphate: NADPH oxidoreductase (III) have been localized to peroxisomes. Taken from Hajra and Bishop (1982).

lack of other lipid biosynthesizing enzymes of this pathway in peroxisomes led to the suggestion that the DHAP pathway enzymes may have functions other than those for the biosynthesis of glycerolipids (Hajra and Bishop, 1982). However, it is now thought that there is cooperation between peroxisomes and microsomes in the biosynthesis of ether glycerolipids with the inital steps performed in peroxisomes and the final steps in the ER. A mechanism of transfer of lipid intermediates is thought to exist between these two organelles (Zaar *et al*, 1987).

### 1.2.5 The Glyoxylate Cycle

The glyoxylate cycle is responsible for the net conversion of acetyl-CoA, derived from fatty acid oxidation, to carbohydrate (Fig. 1.2.6). In the glyoxylate cycle, 2 moles of acetyl-CoA produced by fatty acid oxidation are converted to one mole of succinate, which permits gluconeogenesis (Beevers, 1982). The oxidation of succinate to malate occurs in the mitochondria, and the reactions of gluconeogenesis from oxaloacetate to glucose occur in the cytosol. The enzymes of the glyoxylate cycle were first identified in microbodies from endosperm tissue of germinating castor beans, and hence these organelles are referred to as glyoxysomes (Breidenbach and Beevers, 1967). Since that time, microbodies of unicellular organisms, certain nematodes and plants have been shown to possess the glyoxylate cycle

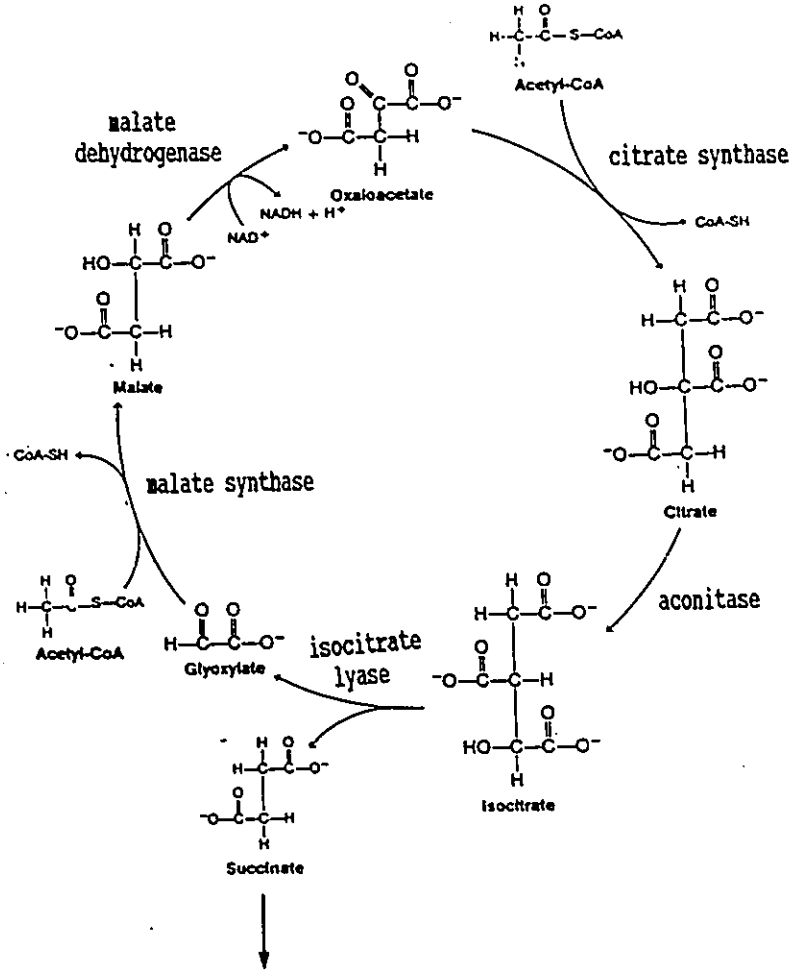



Fig. 1.2.6. The glyoxylate cycle. Adapted from Zubay (1983)

(Goodman *et al*, 1980; Davis *et al*, 1989). All five enzymes of the glyoxylate cycle are found in plant glyoxysomes, whereas the microbodies of other cells (*Tetrahymena* and *Candida tropicalis*) contain only isocitrate lyase and malate synthetase (Lazarow and Fujiki, 1985). Tissues of higher animals were not thought to be able to convert acetyl-CoA into sugars (Jones, 1980). However, the glyoxylate cycle enzymes and the ability to synthesize carbohydrate from lipid have been identified in toad bladder (Goodman *et al*, 1980), fetal guinea pig liver (Jones, 1980) and rat liver (Davis, 1989). As yet, the subcellular locations of the glyoxylate cycle enzymes have not been determined in higher animals.

### 1.2.6 The Pentose Phosphate Pathway

The pentose phosphate pathway is illustrated in Fig. 1.2.7. In this pathway, glucose-6-phosphate is oxidized to ribulose-5-phosphate with the formation of the reduced coenzyme NADPH. From ribulose-5-phosphate, related pentose phosphates can be generated which may be interconverted to a number of three-, four-, five-, six- and seven-carbon sugar phosphates. A recent study observed that the $NADP^+$-dependent dehydrogenases of the pentose phosphate pathway (glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase) are present in rat liver peroxisomes as well as in the cytosol (Antonenkov, 1989). Antonenkov (1989) suggests that the peroxisomal pentose-phosphate pathway dehydrogenases may

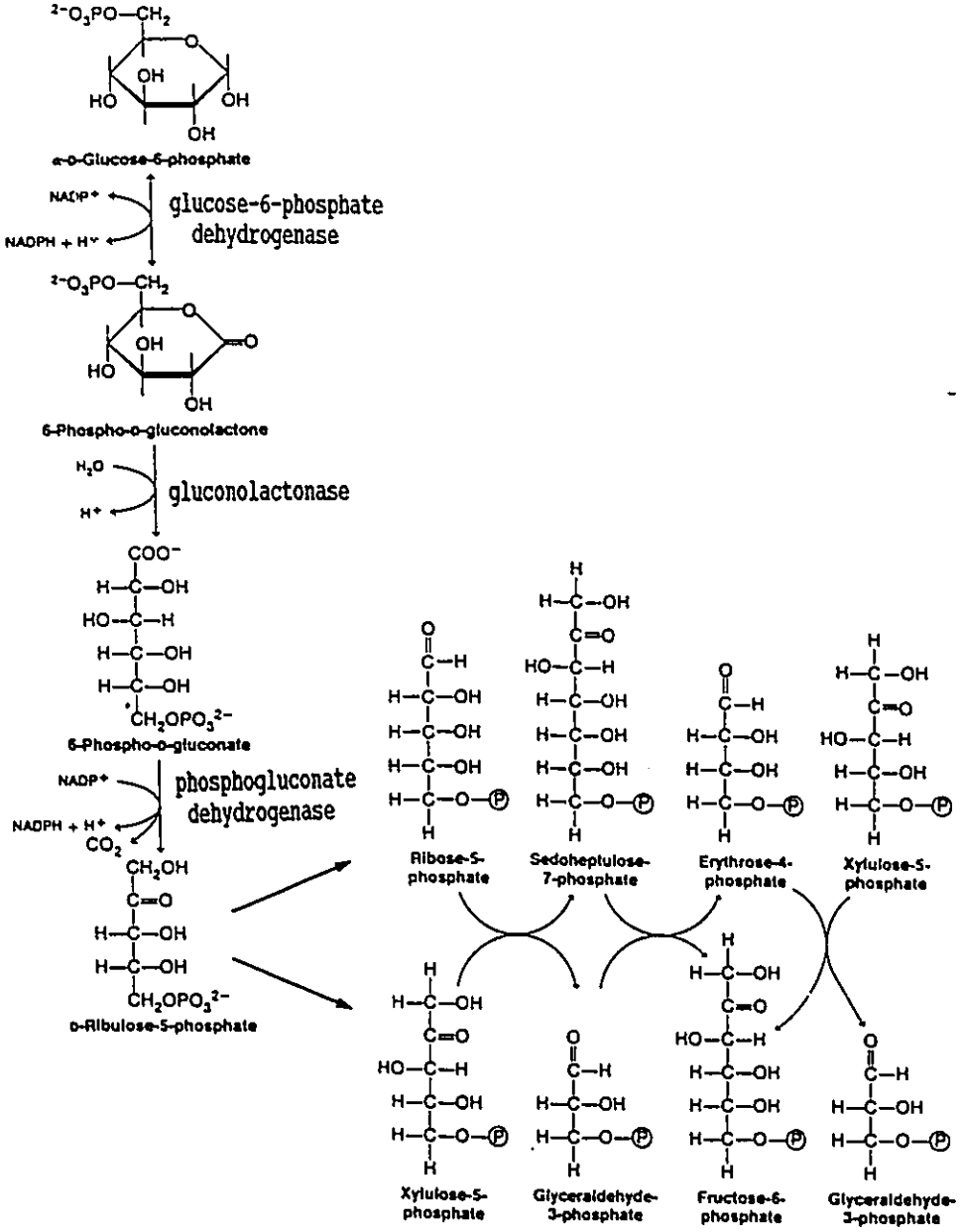



Fig. 1.2.7. The pentose phosphate pathway. Glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase have been localized to rat liver peroxisomes. Adapted from Zubay (1983).

provide the NADPH necessary for peroxisomal lipid synthetic processes.

### 1.2.7 Purine Catabolism

The degradation pathway of purines is shown in Fig. 1.2.8. The nitrogenous excretory products resulting from purine degradation differ between species (Mannaerts and van Veldhoven, 1990). The degradation of purines to urate is common to all animal species, whereas the degradation of urate is much less complete in higher animals. The final enzyme of this pathway present in primates, including man, in birds and in some reptiles is xanthine oxidase, which oxidizes hypoxanthine to xanthine and xanthine to uric acid (Mannaerts and van Veldhoven, 1990). Most mammals (excluding man and new world monkeys) and some reptiles possess urate oxidase, and therefore the end product of purine degradation is allantoin (Masters and Holmes, 1977; Mannaerts and van Veldhoven, 1990). Some fish and amphibians possess allantoinase, and therefore they excrete allantoic acid, while most fish and amphibians have the enzyme allantoicase which hydrolyzes allantoic acid to urea and glyoxylate (Noguchi *et al*, 1979; 1986; Mannaerts and van Veldhoven, 1990). The marine invertebrates retain urease activity and excrete ammonia (Noguchi *et al*, 1979).

Intracellular distribution studies have shown that part of the purine degradation pathway occurs in peroxisomes of animals and plants (Masters and Holmes, 1977). Urate

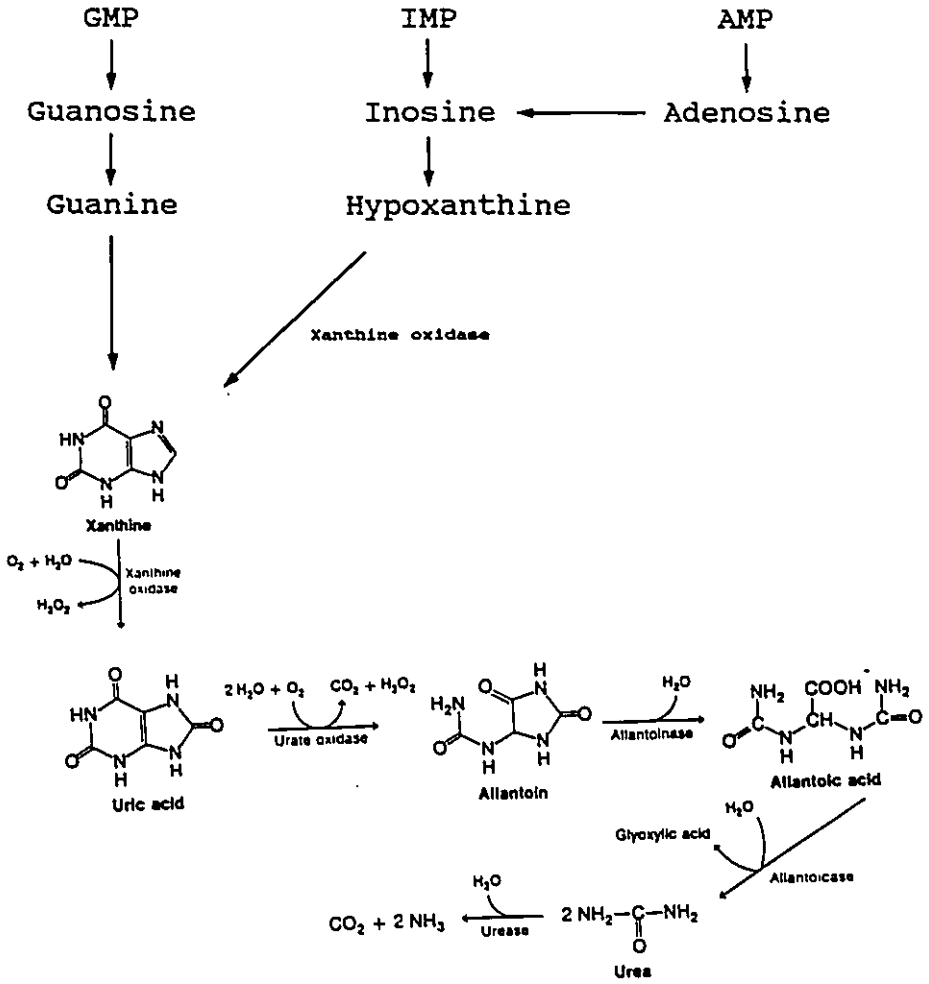



Fig. 1.2.8. Pathways of purine degradation. Adapted from Zubay (1983).

oxidase has been localized to peroxisomes where it is often associated with the paracrystalline core (Masters and Holmes, 1977; Hayashi *et al* 1975). In fish and marine invertebrates both allantoinase and allantoicase are peroxisomal (Noguchi *et al*, 1979; 1986).

For the most part, it appears that enzymes involved in the degradation of purines to urate are located in the cytosol whereas enzymes responsible for the breakdown of urate, when present, are in the peroxisomes (Noguchi *et al*, 1979; 1986).

### 1.2.8 Amino Acid Metabolism

Lysine catabolism in mammals can occur by either the saccharopine pathway or the L-pipecolate pathway (Fig. 1.2.9). The major degradative pathway of lysine proceeds via saccharopine, except in the brain where lysine degradation proceeds mainly via L-pipecolic acid (Wanders *et al*, 1988; Mannaerts and van Veldhoven, 1990). L-pipecolate oxidase is a peroxisomal enzyme, at least in man, and L-pipecolate accumulates in the body fluids of patients with certain peroxisomal diseases (Wanders *et al*, 1988).

The conversion of glutaryl-CoA to glutaconyl-CoA is a common step in the degradation pathways of lysine, hydroxylysine and tryptophan (Vamecq *et al*, 1985; Mannaerts and van Veldhoven, 1990). This reaction is catalyzed by glutaryl-CoA dehydrogenase in the mitochondria and also by glutaryl-CoA oxidase in peroxisomes (Vamecq *et al*, 1985;

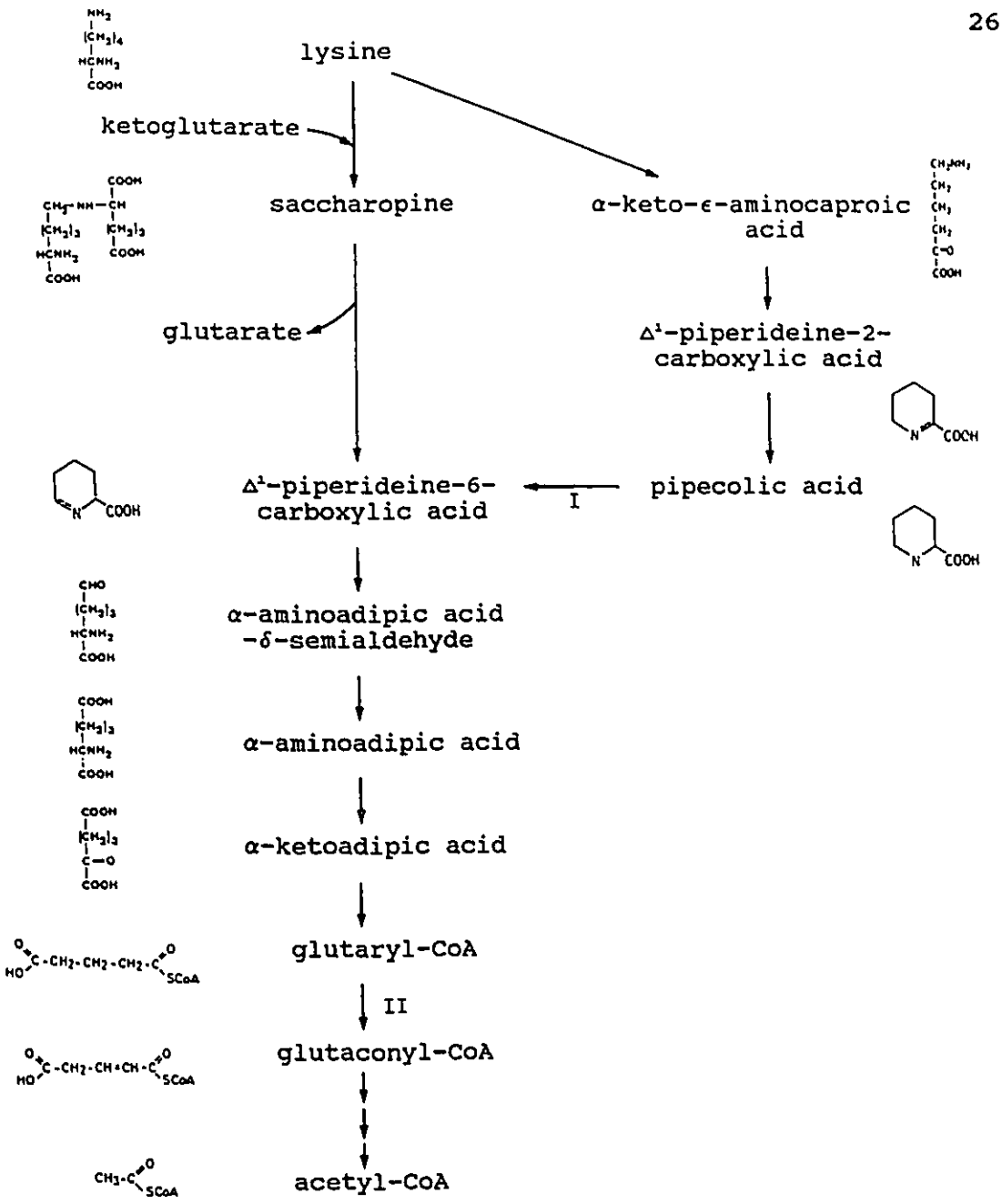



Fig. 1.2.9. Two possible pathways of lysine catabolism. Pipecolate oxidase (I) and glutaryl-CoA oxidase (II) are located in mammalian peroxisomes. Adapted from Hutzler and Dancis (1968), Scriver and Rosenberg (1973) and Vamecq *et al* (1985).

Mannaerts and van Veldhoven, 1990).

Alanine:glyoxylate aminotransferase catalyzes most of the glyoxylate transamination in mammalian tissues with gloxylate as an amino acceptor and primarily alanine as an amino donor, leading to the formation of glycine and pyruvate (Fig. 1.2.10) (Takada *et al*, 1984; Noguchi, 1987; Mannaerts and van Veldhoven, 1990). Two forms of alanine:glyoxylate aminotransferase, designated AGT1 and AGT2, are present in mammalian liver. In certain species (rat and mouse liver) AGT2 predominates, while in dog and cat AGT1 predominates, and human liver contains only AGT1 (Noguchi, 1987). AGT2, when present, is located exclusively in the mitochondria, whereas the subcellular location of AGT1 varies between species (Noguchi, 1987; Takada *et al*, 1984). AGT1 is located in peroxisomes in human, monkey, rabbit and guinea-pig, in mitochondria in dog and cat, and in both organelles in rat, mouse and hamster (Noguchi, 1987).

Glyoxylate is a direct precursor of oxalate via a reaction catalyzed by lactate dehydrogenase or L-α-hydroxyacid oxidase (glycolate oxidase), which is also a peroxisomal enzyme (Fig. 1.2.10) (Mannaerts and van Veldhoven, 1990). Humans with a defect in peroxisomal alanine:glyoxylate aminotransferase exhibit an increased synthesis and excretion of oxalic acid, a disorder called primary hyperoxaluria Type 1 (Williams and Smith, 1978).

The peroxisomal enzyme glycolate oxidase is not only

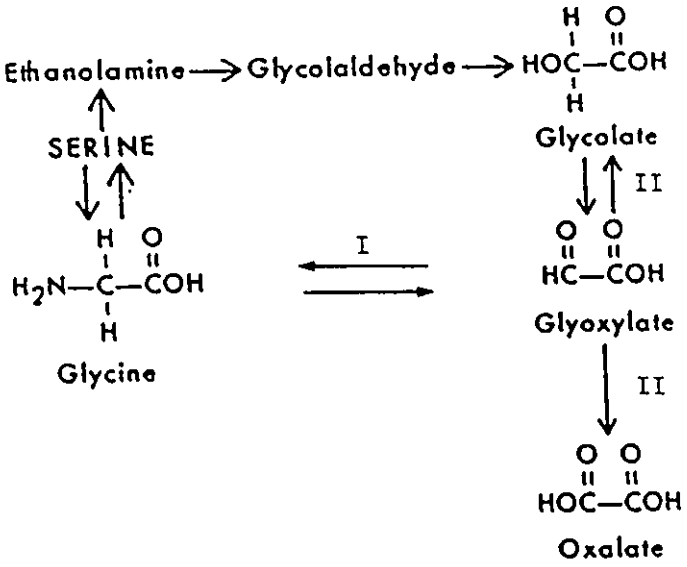



Fig. 1.2.10. Metabolism of glyoxylate in the peroxisomes of some mammals. I, alanine:glyoxylate aminotransferase: II, glycolate oxidase. Adapted from Williams and Smith (1978).

responsible for the conversion of glyoxylate to oxalate but also catalyzes the formation of glyoxylate from glycolate, a breakdown product of ethanolamine (Fig. 1.2.10) (Mannaerts and van Veldhoven, 1990; Williams and Smith, 1978).

Mammalian peroxisomes contain D-amino-acid oxidase, which performs the oxidative deamination of D-amino acids (de Duve and Baudhuin, 1966). D-amino acid oxidase exhibits an absolute stereospecificity for the D-enantiomorph of amino acids but shows a broad substrate specificity, oxidizing both neutral and basic amino acids (Masters and Holmes, 1977; Mannaerts and van Veldhoven, 1990). D-aspartate oxidase oxidizes the D-isomers of acidic amino acids and may also be located in peroxisomes of mammals (Mannaerts and van Veldhoven, 1990). Although D-amino acids from microbial origin may be absorbed to a certain extent from the gut, the physiological functions of these enzymes remain unclear (Mannaerts and van Veldhoven, 1990).

### 1.2.9 Polyamine Catabolism

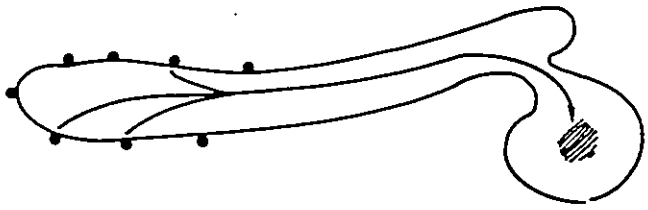
Polyamine oxidase activity responsible for the catabolism of polyamines with the production of $H_2O_2$ has been localized to liver peroxisomes (Hölttä, 1977; Tolbert, 1981).

## 1.3 Peroxisome Biogenesis

### 1.3.1 Biogenesis

Although much is known about the biochemical functions of mammalian peroxisomes, peroxisome biogenesis is less well understood. Many models of peroxisome biogenesis have been proposed over the past years. Early morphological evidence from electron micrographs of rat hepatocytes appeared to show continuity between peroxisomes and the endoplasmic reticulum (ER) (Novikoff and Novikoff, 1982), suggesting that peroxisomes form by budding from the ER. This classical model of peroxisome biogenesis envisioned that peroxisomal proteins are synthesized on membrane-bound polysomes, co-translationally translocated into the ER, and channeled through the ER into buds, which then pinch off to form peroxisomes (Fig. 1.3.1). Early biochemical investigations showed that newly synthesized catalase was apparently found in rat liver microsomes (Higashi and Peters, 1963) supporting this idea. However, later morphological investigations reported that direct peroxisome/ER connections are not seen in rat liver (Legg and Wood, 1970; Rigatuso *et al*, 1970), and it was suggested that new peroxisomes form by budding from existing peroxisomes. Further biochemical studies also supported this theory, as it was demonstrated that there is no detectable diffusion of cytochemical reaction products of catalase and glucose 6-phosphatase between peroxisomes and ER

Model 1: Classical model of peroxisome biogenesis (Lazarow et al, 1982).



Model 2: Current model of peroxisome biogenesis (Lazarow and Fujiki, 1985).

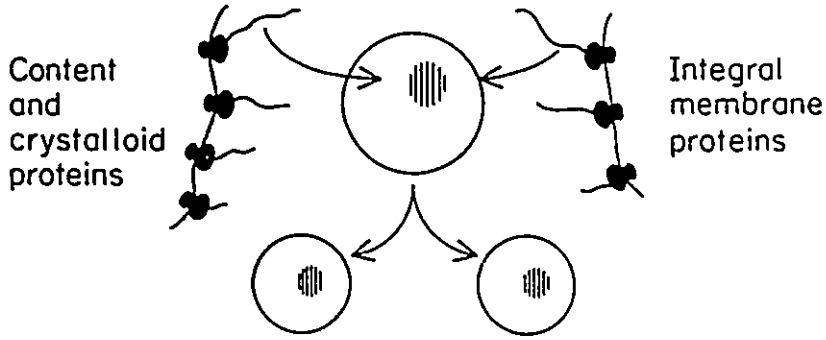



Fig. 1.3.1. Two models of peroxisome biogenesis. Taken from Lazarow *et al* (1982b) and Lazarow and Fujiki (1985).

(Shio and Lazarow, 1981) and that newly synthesized catalase does not pass through the ER en route to peroxisomes (Lazarow and de Duve, 1973; Goldman and Blobel, 1978).

More recently, biochemical studies have demonstrated that all peroxisomal matrix and membrane proteins studied are synthesized on free polysomes, most at their mature size, and are posttranslationally imported into peroxisomes. Finally, Fujiki *et al*, (1982b) found that even though the lipid compositions of the peroxisomal and ER membranes are similar, the polypeptide composition of the ER membrane does not resemble that of the peroxisomal membrane. Taken collectively these data support the second model of peroxisome biogenesis which proposes that peroxisomes grow by the posttranslational incorporation of new proteins and that new peroxisomes arise from division of existing peroxisomes (Fig. 1.3.1).

A third model of peroxisome biogenesis is a compromise between the first two models. This model proposes that at least one of the peroxisomal membrane proteins is synthesized on membrane-bound polysomes and is co-translationally inserted into the rough ER, where it is sorted from the other integral membrane proteins to form a patch that buds from the ER and gives rise to an immature peroxisome (Goldman and Blobel, 1978; Köster *et al*, 1986). Subsequently, the remaining peroxisomal matrix and membrane proteins, synthesized on free polysomes, are inserted into the immature peroxisome to form the mature organelle (Goldman and Blobel, 1978; Köster *et al*,

1986). Thus far, there is little evidence to support this model.

Our current knowledge of peroxisome biogenesis is consistent with the second model and can be summarized as follows:

1) peroxisomal proteins are encoded by nuclear genes
2) peroxisomal proteins are synthesized on free polysomes and posttranslationally imported into peroxisomes
3) most peroxisomal proteins are synthesized at their mature size and do not undergo any posttranslational modification (*e.g.* glycosylation) (Lazarow and Fujiki, 1985; Borst, 1986).

### 1.3.2 Turnover of Peroxisomes

In the maintenance of cellular organelles, synthesis and degradation must be considered as a continuum. It was originally concluded by de Duve and Baudhuin (1966) that peroxisomes grow continuously for a period of about 4 days before they burst, releasing their contents into the cytoplasm where they would be rapidly destroyed. In contrast, Poole *et al* (1969) calculated the half-life of peroxisomes to be between 1.5 and 2.5 days and found that proteins lose radioactivity incorporated from labeled precursors in an exponential fashion, suggesting peroxisomes are destroyed in a random way. In addition, it was shown that all major protein components of the peroxisome have the same rate of

turnover, which suggested that the particles are destroyed *in toto*, perhaps by autophagy (Poole *et al*, 1969). Subsequently, Poole *et al* (1970) studied the incorporation of labeled catalase into peroxisomes of different sizes and found no influence of particle size on the specific radioactivity of catalase. This suggested that peroxisomes do not exist as independent individuals but continuously (or intermittently) exchange material with one another (Poole *et al*, 1970). Poole *et al*, (1970) further suggested that this could occur by continual fission of particles followed by growth, which is consistent with our present understanding of peroxisome biogenesis.

Little is known of the actual mechanism of destruction of peroxisomes, but it has been suggested that they are subject to autophagy by lysosomes (Poole *et al*, 1969; Pfeifer *et al*, 1978). Although some studies have not found evidence of this, Svoboda *et al* (1967) observed some microbodies engulfed by lysosomes in morphological analyses. Svoboda *et al* (1967) noticed that the occurrence of microbodies in lysosomes is too rare to account for their entire degradation and suggested that there may be other mechanisms in addition to autophagy. However, other mechanisms of microbody degradation are not well defined.

### 1.3.3 Protein Targeting to Peroxisomes

Most peroxisomal proteins are not synthesized as

larger precursors and do not undergo posttranslational modifications. Therefore, the information for targeting these proteins to the organelle must reside in the mature proteins. As the sequences for genes encoding peroxisomal proteins have been determined, they have been used to analyze the role of specific sequences in the targeting of proteins to peroxisomes. Using a variety of experimental approaches, the natures of the peroxisomal targeting signals and of the import process are just beginning to be elucidated.

Subramani and co-workers made the surprising observation that firefly luciferase is located in peroxisomes when its cDNA is expressed in mammalian cells (Keller *et al*, 1987). Using luciferase as a model protein, Gould *et al* (1987) identified regions of this protein which are necessary for its targeting to peroxisomes. Using site-directed mutagenesis, Gould *et al* (1988; 1989) determined that the carboxy-terminal three amino acids of luciferase are both necessary and sufficient to direct luciferase or other reporter proteins into peroxisomes. These three amino acids located at the carboxy-terminus have the sequence S-K-L and are referred to as the tripeptide peroxisomal targeting signal (PTS) (Gould *et al*, 1988; 1989). In this PTS the last amino acid (L) is invariant and cannot be removed or replaced by even I or V without loss of specific targeting function (Gould *et al*, 1988; 1989). The K can be replaced by R or H, suggesting that the only requirement at this position is a

basic amino acid, and the S can be replaced by other small amino acids such as A or C (Gould *et al*, 1988; 1989). From these studies it appears that the minimal topogenic sequence is S/A/C - K/R/H - L.

Although SKL variants are sufficient for targeting proteins to peroxisomes, the context in which the tripeptide is placed also plays a role. This is evident from the observation that some deletions in the amino-terminus of luciferase block import even though the carboxy-terminal tripeptide remains intact (Gould *et al*, 1987). This probably means that the SKL sequence must be exposed to allow interaction with a putative receptor, and alterations in the protein which render the signal inaccessible would abolish import (Borst, 1989). This would explain why many cellular proteins that contain an SKL somewhere in their sequence are not targeted to peroxisomes (Gould *et al*, 1988).

In support of the findings by Gould *et al* (1987; 1988; 1989), *in vitro* import studies using purified rat liver peroxisomes have localized a PTS within the last five amino acids of rat acyl-CoA oxidase, which contains SKL at the carboxy-terminus (Miyazawa *et al*, 1989). In addition, a number of peroxisomal proteins have been found to possess the carboxy-terminal tripeptide PTS (Borst, 1989), and there is evidence to suggest that the SKL motif may be a universal signal in peroxisome targeting. Experiments with firefly luciferase have shown that the SKL motif is sufficient for

targeting proteins to microbodies of insects, mammals, plants and yeast (Gould *et al*, 1990a). These results demonstrate a high degree of conservation of the targeting signal and the import machinery.

On the other hand, not all peroxisomal proteins possess the tripeptide PTS at their carboxy-terminus and some do not contain this tripeptide at all (Borst, 1989). It is possible that the conserved tripeptide PTS can act at an internal location, as appears to be the case for human catalase which possesses an S-H-L tripeptide 11 to 9 amino acids from the carboxy-terminus (Gould *et al*, 1988). Alternatively, there may be more than one type of PTS (Borst, 1989). In this respect, *in vitro* studies have identified two regions of *C. tropicalis* acyl-CoA oxidase (POX 4) which are able to target it to peroxisomes (Small *et al*, 1988), and neither of these regions contains a variation of the tripeptide PTS (Borst, 1989).

Another approach to searching for targeting signals is to compare isoenzymes which are targeted to distinct intracellular compartments. This approach has been utilized in the case of cytoplasmic and glycosomal phosphoglycerate kinases (PGK) of *Trypanosoma brucei* and *Crithidia fasciculata* (Osinga *et al*, 1985; Swinkles *et al*, 1988). The two enzymes are highly homologous, except that the glycosomal PGK possesses a carboxy-terminal extension which has a net positive charge and is thought to play a role in glycosomal

targeting (Osinga *et al*, 1985; Swinkles *et al*, 1988). Similarly, mammalian peroxisomal 3-ketoacyl-CoA thiolase has a cleavable amino-terminal sequence which is absent from the mitochondrial isoenzyme (Arakawa *et al*, 1987; Hijikata *et al*, 1987). Evidence that this amino-terminal extension is involved in peroxisomal targeting is presented later in this thesis (Results and Discussion, section 4.7). Therefore, in addition to the carboxy-terminal tripeptide (SKL), there appear to be other types of PTSs. The characteristics of the other PTSs are not yet defined, and it is not known if they use different components of the import machinery.

Although research on peroxisome biogenesis has lagged behind that of mitochondria and chloroplasts, these organelles provide us with a plausible working model for targeting to peroxisomes (Pugsley, 1989). Peroxisomal proteins contain uncleaved sequences which route them specifically to the peroxisome surface, where they recognize and bind to specific surface receptors and are subsequently imported (Pugsley, 1989). Following import, peroxisomal proteins may undergo conformational changes (*e.g.* refolding or oligomerization), which may be important for retention of proteins in the organelle (Pugsley, 1989). Peroxisomal proteins have been shown to bind specifically to peroxisomes and not to mitochondria *in vitro*, supporting the notion that there are specific receptors on their surface (Imanaka *et al*, 1987; Small *et al*, 1987). The *in vitro* import process appears to be

energy dependent as it requires ATP hydrolysis (Imanaka *et al*, 1987). However, it is not clear whether ATP is necessary to drive protein import or to unfold proteins rendering them import competent (Pugsley, 1989).

Little is known about the possible cytoplasmic or membrane-bound components that are involved in targeting to peroxisomes. The use of reliable *in vitro* assay systems, as well as characterization of mutants defective for peroxisomal protein import (see Introduction, section 1.7), will undoubtedly facilitate elucidation of the requirements for protein import and characterization of the import machinery.

### 1.3.4 Protein Targeting to Other Cellular Organelles

Eukaryotic organelles are highly specialized for their particular functions. To maintain this organization cells must efficiently direct proteins to their correct subcellular destinations. Although not much is known about protein targeting to peroxisomes, protein targeting to the ER (secretory pathway), mitochondrion, chloroplast and nucleus is fairly well characterized. In this section, the targeting of proteins to these organelles will be discussed for comparison to protein targeting to the peroxisome. For a more comprehensive review please refer to Pugsley (1989).

#### 1.3.4.1 Targeting of Proteins to Mitochondria

Mitochondria have four compartments to which proteins

must be correctly targeted: the outer and inner membranes, the intermembrane space and the matrix. The majority of mitochondrial proteins (>90%) are encoded by nuclear genes, synthesized on free polysomes and are posttranslationally imported (Hallermayer *et al*, 1977; Hay *et al*, 1984). In most cases mitochondrial proteins are synthesized as precursors, containing cleavable amino-terminal extensions which are responsible for directing the proteins to mitochondria (Hurt *et al*, 1984; van Loon and Young, 1986). A remarkable feature of the amino-terminal prepiece is the lack of similarity between signals from different precursor proteins (von Heijne, 1986). The prepieces vary in both size and amino acid sequence (von Heijne, 1986). The prepieces generally possess a high content of basic amino acids, a few hydrophobic residues, a preponderance of hydroxylated residues and an absence of acidic residues (von Heijne, 1986). Analysis of a number of mitochondrial prepieces revealed that they could form amphipathic α-helices, with the basic amino acids on one face of the helix and the hydrophobic residues along the other face (Roise *et al*, 1986; von Heijne, 1986).

Proteinaceous receptors have been identified on the outer mitochondrial membrane that are involved in the specific recognition of the precursors (Zimmermann *et al*, 1981; Zwizinski *et al*, 1984; Pfaller and Neupert, 1987). Import of precursors into the mitochondria occurs in a single step at contact sites between the inner and outer membrane (Schleyer

and Neupert, 1985, Schwaiger *et al*, 1987). The insertion into and translocation across the outer and inner membranes requires an electrochemical potential across the inner membrane (Pfanner and Neupert, 1986). It has been postulated that this electrochemical membrane potential produces an electrophoretic driving force on the positively charged residues of the precursor protein, which may mediate insertion into or through the inner membrane (Pfanner and Neupert, 1986; Roise *et al*, 1986). It is significant that insertion of some proteins (*e.g.* porin) into the outer mitochondrial membrane is independent of a transmembrane electrochemical potential (Gasser and Schatz, 1983). In addition to a transmembrane electrochemical gradient, protein import requires ATP or GTP (Pfanner and Neupert, 1986; Pfanner *et al*, 1987). It is thought that the hydrolysis of NTPs may be required to unfold proteins into an import competent state (Pfanner *et al*, 1987). Recent studies have also implicated one of the heat shock family of proteins (HSP70) in preventing tight folding of the precursors in the cytosol (Deshaies *et al*, 1988).

During or immediately following translocation through the membranes, the precursor proteins with amino-terminal prepieces are proteolytically processed by a peptidase located in the matrix (Miura *et al*, 1982; Hawlitschek *et al*, 1988). Once directed to mitochondria, imported proteins must be correctly sorted to their appropriate intramitochondrial location. Some mitochondrial inner membrane proteins and

proteins of the intermembraneous space are imported entirely into the matrix and then reinserted into or through the inner membrane from the inner face (Hartl *et al*, 1987), while others appear to remain membrane-associated (van Loon and Schatz, 1987). Proteins of the intermembraneous space are thought to be processed in two stages, first by the matrix peptidase and then by an enzyme located in the intermembraneous space (Hartl *et al*, 1987).

Recent studies have focussed on characterization of the key components of the mitochondrial import system, including the cytosolic and intramitochondrial factors involved in transport, processing and folding of proteins (Baker and Schatz, 1991).

1.3.4.2 Targeting of Proteins to Chloroplasts

The chloroplast contains six different compartments to which proteins can be targeted. In addition to the outer and inner membranes, stroma (the equivalent of the mitochondrial matrix) and intermembrane space, the chloroplast contains membrane-delimited thylakoids within the stroma. The majority of chloroplast proteins are encoded by nuclear genes, synthesized as precursors on free polysomes and posttranslationally imported into chloroplasts (Ellis, 1981).

All known chloroplast proteins possess amino-terminal cleavable presequences. Evidence from gene-fusion studies indicates that the presequences carry specific targeting

information for the chloroplast (Schreier *et al*, 1985; van den Broeck *et al*, 1985). The prepieces vary widely in size (30-80 amino acids) and sequence; however, all include a high proportion of basic and hydroxylated amino acids with few acidic residues (von Heijne *et al*, 1989). Analysis of known stroma-targeting peptides reveals a conserved three-domain design: an amino-terminal uncharged domain, a central positively charged domain of varying length and a carboxy-terminal domain that has a high potential for forming an amphipathic ß-strand (von Heijne *et al*, 1989).

The precursors are thought to bind to receptors on the outer chloroplast membrane and are imported into the chloroplasts at sites where the inner and outer membranes are juxtaposed (Kaderbhai *et al*, 1988; Pain *et al*, 1988). The chloroplasts use ATP as the sole source of energy for the import process (Flügge and Hinz, 1986). Precursors of stromal proteins are processed to their mature sizes by a stromal peptidase during or shortly after translocation (Robinson and Ellis, 1984a; 1984b). The sorting of chloroplast proteins to their appropriate subcompartments is not well defined. Import into the thylakoid lumen is signalled by composite transit peptides with a stroma-targeting amino-terminal part and a thylakoid-targeting carboxy-terminal segment (von Heijne *et al*, 1989).

Both mitochondria and chloroplasts have endogenous genomes located in the matrix (or stroma) encoding some

proteins which require transport into or across either the inner membrane or the thylakoid membranes (in chloroplasts). Little is known about targeting of endogenous proteins in these organelles (Pugsley, 1989).

1.3.4.3 The Targeting of Nuclear Proteins

The nucleus is surrounded by a double bilayer membrane that is continuous with the endoplasmic reticular membrane, and therefore they share many lipids and proteins (Pugsley, 1989). However, targeting of nuclear proteins is independent of the secretory pathway (Pugsley, 1989). Accumulation of proteins in the nucleus depends on internal signals within protein sequences. Signals which define nuclear localization were first identified by Kalderon *et al*, (1984a) using the large T antigen of SV40. Kalderon *et al*, (1984a) found a signal of SV40 T antigen located close to the centre of the polypeptide which, when fused to ß-galactosidase or pyruvate kinase, is capable of directing these proteins to the nucleus. The nuclear localization signal has been reduced to the sequence -Pro-Lys-Lys-Lys-Arg-Lys-Val (Kalderon *et al*, 1984b). The signals that have been identified from different nuclear proteins do not exhibit significant sequence similarity, but share general characteristics (Pugsley, 1989). The sequences possess an overall basic charge due to the presence of several lysine residues and some arginine residues, as well as a ß-turn-inducing proline or glycine residue (Pugsley, 1989).

The nuclear envelope is perforated by pores which play a role in the movement of macromolecules across the envelope (Newport and Forbes, 1987). The pore is thought to play a central role in the import of proteins into the nucleus, as labeled nuclear proteins have been found to be associated with the pores (Feldherr *et al*, 1984; Dworetzky *et al*, 1988). *In vitro* studies have determined that nuclear protein import is an ATP-dependent process (Newmeyer *et al*, 1986); however, the exact role of ATP in nuclear protein import has not been determined.

Recently, several groups have used a synthetic nuclear signal peptide to identify cellular components that are involved in import into the nucleus. Putative receptors have been localized to the cytoplasm, nuclear envelope and nuclear interior (Adam *et al*, 1989; Silver *et al*, 1989). It has been suggested that these proteins act sequentially to transport the protein from the cytoplasm, through the nuclear pore and finally to the interior of the nucleus (Adam *et al*, 1989).

#### 1.3.4.4 Targeting Proteins to the Secretory Pathway

In eukaryotic cells, proteins enter the secretory pathway at the rough ER membrane and travel through or are retained by the compartments of this pathway (ER, Golgi, secretory (storage) granules, lysosomes or plasma membrane). Biogenesis of proteins of the secretory pathway is quite well characterized (Verner and Schatz, 1988; von Heijne, 1988;

Pugsley, 1989). With few exceptions [cytochrome $b_5$ (Rachubinski *et al*, 1980), cytochrome $b_5$ reductase (Borgese and Gaetani, 1980), the RAS-like GTP binding proteins and the mating pheromones of some yeasts (Powers *et al*, 1986)] the proteins of the secretory pathway are synthesized on membrane-bound polysomes and are co-translationally translocated into the ER (Verner and Schatz, 1988; von Heijne, 1988; Pugsley, 1989). Most of these proteins are synthesized with amino-terminal signal sequences which direct the protein to the ER and are subsequently cleaved to give the mature protein (Pugsley, 1989). Although the size and sequence of the amino-terminal signal sequence are quite variable (15-33 amino acids), the general characteristics of the signal peptide are well defined (von Heijne, 1988). Three elements have been distinguished: an amino-terminal part which spans 1-20 amino acids and possesses an overall net positive charge, a middle section (7-16 residues) which is enriched in hydrophobic amino acids and a carboxy-terminal portion that contains a consensus sequence involved in recognition and cleavage by a processing enzyme (signal peptidase) (von Heijne, 1988; Pugsley, 1989). This carboxy-terminal region is generally polar and contains a helix-breaking amino acid (typically proline) adjacent to the hydrophobic core region and approximately six residues upstream from the cleavage site (von Heijne, 1988). The specific cleavage site requires small uncharged residues (typically glycine or alanine) in positions -3 and -1 for

efficient cleavage by signal peptidase (von Heijne, 1988).

The targeting of proteins to the ER membrane requires many factors, including proteins in the cytosol and on the target membrane. Walter and Blobel (1981) and Meyer *et al*, (1982) isolated two receptor proteins involved in the targeting process, the signal recognition particle (SRP) and the SRP receptor. SRP is a ribonucleoprotein particle composed of six nonidentical polypeptides and a 7S RNA, which binds to the signal sequence as it emerges from the ribosome, arresting polypeptide chain growth (Walter and Blobel, 1981; Walter *et al*, 1984). Meyer *et al*, (1982) named the SRP receptor 'docking protein', which they believed to be a 72 kDa integral membrane protein of the rER. It is now known that docking protein is the α subunit of the heterodimeric SRP receptor (Tajima *et al*, 1986; Hortsch and Meyer, 1988). The α subunit is a peripheral membrane protein which may be anchored to the cytoplasmic face of the rER membrane by interactions with the β-subunit, a 30 kDa integral membrane protein (Tajima *et al*, 1986; Hortsch and Meyer, 1988). Upon binding of the SRP-polysome complex to the SRP receptor, the SRP is displaced from the ribosome and chain elongation resumes (Walter and Blobel, 1981; Gilmore and Blobel, 1983). The growing polypeptide chain passes co-translationally through the ER membrane into the lumen, where the signal peptide is cleaved (Verner and Schatz, 1988; Pugsley, 1989).

The mechanism of translocation of the polypeptide

across the ER membrane is not resolved. It is not known whether translocation is directly through the lipid environment or through proteinaceous pores. Engelman and Steitz (1981) argued on thermodynamic grounds that protein translocation could occur without the involvement of membrane proteins. However, there are strong theoretical arguments and experimental evidence for the translocation of secretory polypeptides through an aqueous channel (Gilmore and Blobel, 1985; Singer *et al*, 1987a; 1987b). Singer *et al*, (1987a; 1987b) proposed that specific 'translocase' proteins form a channel through which soluble proteins could pass and from which hydrophobic membrane-anchoring domains could dissociate, to be released into the lipid environment. In support of this proposal, Gilmore and Blobel (1985) found evidence that nascent translocation intermediates are accessible to aqueous reagents. Furthermore, Wiedmann *et al*, (1987) proposed that the α-subunit of the SRP receptor could form a channel in the ER membrane. Wiedmann *et al*, (1987) introduced photoreactive groups into the signal peptide and, after irradiation, induced crosslinks to the α-subunit of the SRP receptor.

Following translocation into the ER lumen, a protein in the ER (BiP), which is structurally related to heat shock proteins, plays a role in ensuring that proper folding and oligomerization of the translocated protein occur (Munro and Pelham, 1986).

The sorting of proteins within the secretory system

(ER, Golgi, secretory vesicles, lysosomes or plasma membranes) will only be discussed briefly as the early events of import into the ER are the most relevant to the present discussion. For a comprehensive overview, please refer to Pugsley (1989).

There are many factors involved in determining which proteins pass unimpeded through the secretory pathway and which are retained in the various compartments. There are two general models to explain this phenomena. The first model proposes that all secretory proteins have signals which target them successively through the secretory pathway to their final destination. In this model, proteins are retained in certain organelles if they lack the signal necessary for targeting to the next organelle in the pathway (Pugsley, 1989). Conversely, the second model suggests that secretory proteins travel through the pathway in the bulk flow unless they have specific retention signals causing them to be retained by specific compartments (Pugsley, 1989). It is not clear which of these mechanisms is correct or if specific transport depends on a combination of both models. The second model is supported by the identification of an ER retention signal (amino acids K-D-E-L) which has been identified at the carboxy-termini of a number of ER proteins (Munro and Pelham, 1987).

In addition to the nature of the signals which determine a protein's ultimate destination, another interesting aspect of the secretory pathway is how proteins

move between the successive compartments. The various compartments of the secretory pathway beyond the rER are structurally independent, and proteins move between them in specific classes of transport vesicles (Pugsley, 1989). These membrane-enclosed vesicles bud from the donor membrane carrying soluble or membrane proteins and bind to the target membrane, presumably through a receptor mediated interaction (Pugsley, 1989). This process may involve a recycling mechanism whereby receptors (and possibly missorted proteins) can be returned to their appropriate locations to ensure the fidelity of the system (Pugsley, 1989).

1.3.4.5 General Comments on Protein Targeting

Although the signals that target proteins to the various intracellular compartments are quite distinct, there seems to be a common sequence of events involved in all the import processes. All the pathways require ATP, cytosolic components, membrane proteins and intraorganellar factors. These components play a successive role in the import process. Cytosolic factors act to keep the polypeptides in an import-competent state, the membrane-bound receptors specifically bind the signal and may mediate translocation across the membrane(s) and intraorganellar factors play a role in processing, proper folding and/or maturation of the protein within the organelle. Although the components of the peroxisomal import machinery are just beginning to be

elucidated, the other pathways serve as excellent working models for these investigations, and transport of proteins into peroxisomes may certainly involve a series of similar functionally defined steps.

Protein targeting signals can be classified into two groups. The first group includes signals located at the amino-termini of proteins which are generally long and loosely defined and are removed from the proteins after (or during) import (*e.g.* ER, mitochondria, chloroplasts). The second group is characterized by signals which are non-cleavable, relatively short and less variable in sequence (*e.g.* peroxisomes, nuclei). Why certain organelles use these different types of signals is not understood. Future studies will undoubtedly involve further characterization of the signals and components involved in the import processes and the actual mechanism(s) of transport of proteins across membranes.

## 1.4 Evolutionary Origin of Microbodies

It is now widely accepted that microbodies form by growth and division of existing microbodies and that the ER does not play a role in their formation (Lazarow and Fujiki, 1985). If this is the case, where did the first microbody come from? It has been suggested that microbodies are descendants of ancient endosymbionts (de Duve, 1982). de Duve (1982) further proposed that the microbody served as a

primitive respiratory organelle, lacking oxidative phosphorylation, but capable of oxidizing all major foodstuffs in a pre-eukaryotic cell yet devoid of mitochondria. After symbiotic adoption of the aerobic bacterium that was to become the mitochondrion, the peroxisome became more specialized, performing only certain specific functions in various cell types (de Duve, 1982).

It is now accepted that mitochondria and chloroplasts are descendants of ancient endosymbionts (Borst, 1989). However, peroxisomes differ from these organelles in two fundamental respects: they have no DNA and they have only one membrane. de Duve (1983) suggests that these points do not preclude the endosymbiotic origin for microbodies. Both mitochondria and chloroplasts have transferred more than 90% of their genetic material to the nucleus through the course of evolution. de Duve (1983) argues that the peroxisome may have transferred 100% of its genetic material, which is plausible if the organelle was adopted as an endosymbiont at an earlier time. Regarding the absence of a double membrane in peroxisomes, de Duve (1983) observed that some endocytic parasites escape into the cell cytosol and are bounded by only one membrane.

The endosymbiont hypothesis can be tested by comparing the amino acid sequences of microbody proteins (or the nucleotide sequences of the genes encoding them) with the sequences of homologous molecules in various eukaryotic and

bacterial cells (de Duve, 1983). Many genes encoding microbody enzymes have now been sequenced, but there are only a few sequences available for the corresponding enzymes in the eukaryotic cytosol and in bacteria. Most of these sequences have been uninformative, except perhaps those of the glyceraldehyde phosphate dehydrogenases (GAPDH) of *T. brucei* (Borst, 1989). *T. brucei* has cytosolic and glycosomal forms of GAPDH which differ significantly. Comparison of the glycosomal enzyme to other eukaryotic GAPDHs and to the *Escherichia coli* enzyme suggests that the glycosomal enzyme stems from a different lineage than that of the cytosolic enzyme (Borst, 1989). However, much more work is required before one can conclude an endosymbiotic origin of the microbody.

If the microbody was adopted endosymbiotically, it will be of interest to determine if the different classes of microbodies were derived from a common ancestor or whether different endosymbiotic events gave rise to the various classes of microbodies (peroxisomes, glyoxysomes and glycosomes). Evidence for a common origin of microbodies of fungi, plants and animals comes from analysis of the enzymes of the ß-oxidation system (Kunau *et al*, 1988). Kunau *et al* (1988) found remarkable uniformity in the overall organization and subunit structure of the enzymes involved in microbody ß-oxidation, quite distinct from those of the mitochondrial ß-oxidation pathway.

If all microbodies arose from one common ancestor, two explanations for the existence of the diversity in enzyme composition of microbodies can be given (Borst, 1986). The first suggests that microbodies are the ancestors of a very versatile prokaryote acquired endosymbiotically. Differential loss of enzymatic capabilities would result in the differences between the microbodies of different organisms observed today (Borst, 1986). Alternatively, the diverse enzymatic compositions of microbodies could be the result of the redistribution of enzymes over the course of evolution (Borst, 1986). This redistribution undoubtedly involved both the loss and gain of enzyme pathways (Borst, 1986). It is difficult to understand how complex metabolic pathways could have localized to the microbody by the transfer of many enzymes from other cellular locations (Borst, 1989). However, if targeting information is relatively simple (*i.e.* specified by only a few amino acids), it may not be that difficult to relocate enzymes.

Enclosing certain metabolic reactions in microbodies may be advantageous for various reasons (Borst, 1989). Relocation of a complex pathway to microbodies may increase its efficiency by shortening substrate diffusion times or by providing different environmental conditions (*e.g.* pH) for optimal enzyme activity (Borst, 1989). Additionally, relocation may serve to segregate reactions which produce toxic or reactive metabolites for local inactivation or to

partition reaction products to different metabolic fates (Borst, 1989). Alternatively, some enzymes may have been relocated to peroxisomes by random mutations that inadvertently produced microbody targeting signals but that did not have any obvious selective advantage (Borst, 1989).

Further studies on the evolutionary origin of microbodies will determine whether they were acquired by endosymbiosis or by the appearance of a new, independent eukaryotic compartment by superspecialization of a pre-existing one (Borst, 1989).

## 1.5 Induction of Mammalian Peroxisomes by Hypolipidemic Drugs

A remarkable feature of peroxisomes is their ability to proliferate in response to treatment with a number of substances. Treatment of rodents with several hypolipidemic drugs, herbicides and organic plasticizers causes a marked proliferation of liver peroxisomes (Reddy, 1990). Similarly, peroxisomes in other organisms can be induced by growth on suitable substrates. For example, peroxisomes can be induced in certain yeasts by growth on alkanes or fatty acids (*e.g. Candida* species and *Saccharomyces cerevisiae*) or by growth on methanol (*e.g. Hansenula polymorpha*) (Lazarow and Fujiki, 1985). This discussion will be restricted to the effects of hypolipidemic drugs on mammalian cells.

A variety of structurally diverse hypolipidemic drugs,

herbicides and organic plasticizers induce peroxisome proliferation in rodent hepatocytes (Reddy, 1990). The inducibility of peroxisomes has been used both to facilitate the isolation of large quantities of peroxisomes and to study their physiological role. In addition, evidence that chronic exposure to some of these compounds leads to the development of hepatocellular carcinomas in experimental animals has directed attention to understanding the mechanisms of action of these compounds and their potential health risks to humans (Reddy *et al*, 1980).

A list of some peroxisome proliferators is shown in Table 1.5.1. Of these, clofibrate and its analogs are among the most widely used hypolipidemic drugs in clinical medicine (Reddy *et al*, 1980). Administration of clofibrate and other hypolipidemic drugs to rats causes a 4- to 10-fold increase in the number of hepatic peroxisomes in electron microscopic images, with a concomitant increase in peroxisomal ß-oxidation activity (Hess *et al*, 1965; Lazarow and de Duve, 1976; Lazarow, 1977). This increase in ß-oxidation activity is accompanied by an induction of the peroxisomal enzymes involved in ß-oxidation (fatty acyl-CoA oxidase, hydratase dehydrogenase and 3-ketoacyl-CoA thiolase) (Lazarow *et al*, 1982a). It has also been demonstrated that clofibrate causes proliferation of smooth ER and an approximately 10-fold increase in a specific hepatic microsomal isoenzyme, cytochrome P-452 (P-450 IVA1), which has specificity for the

Table 1.5.1 Structures of Peroxisome Proliferators

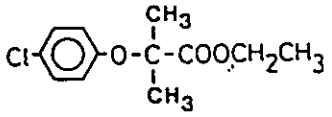
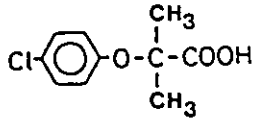
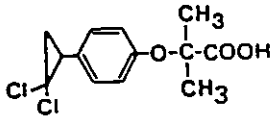
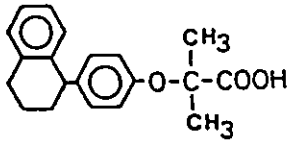
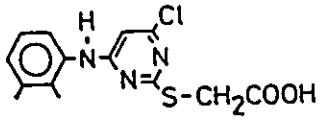
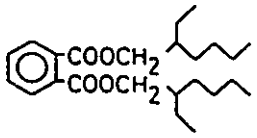
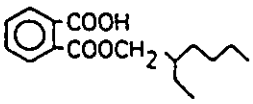
| | |
|---|---|
| **Hypolipidemic Drugs** | |
| Clofibrate |  |
| Clofibric acid<br>(active metabolite of clofibrate) |  |
| Ciprofibrate |  |
| Nafenopin |  |
| Wy-14,643 |  |
| **Plasticizers** | |
| Di(2-ethylhexyl)phthalate (DEHP) |  |
| Mono(2-ethylhexyl)phthalate (MEHP)<br>(active metabolite of DEHP) |  |

Table constructed from Lalwani *et al* (1983) and Lundgren *et al* (1987).

ω-hydroxylation of fatty acids (Sharma *et al*, 1988a). There appears to be a close association between the induction of microsomal ω-hydroxylase activity and the peroxisomal fatty acid ß-oxidation pathway. It has been postulated that enzyme induction in these two hepatic organelles is mechanistically related; however, the relationship remains to be determined (Sharma *et al*, 1988a; 1988b). The effects of peroxisome proliferators on other organelles are not well characterized. Some reports have documented a slight increase in the numbers of lysosomes and mitochondria; however, it is not known if the changes are a primary effect of clofibrate-treatment or a secondary effect. For example, it has been noted that there are slight increases in the number and size of hepatic lysosomes; however, this may be due to an increase in autophagic degradation of peroxisomes by lysosomes (Svoboda *et al*, 1967). Other studies have noted that the mitochondria from clofibrate-treated rat livers are similar to controls in structure and protein content but are increased in number by 2-fold (Lipsky and Pedersen, 1982). There is also an increase in a few specific mitochondrial enzymes, especially those involved in ß-oxidation (*e.g.* carnitine acyl transferase) (Lipsky and Pedersen, 1982). This may be a result of increased mitochondrial ß-oxidation substrates generated by peroxisomes.

The induction of peroxisomal and microsomal enzymes involved in the hypolipidemic effect is due to increased rates

of transcription of their respective genes in hepatocytes (Furuta *et al*, 1982, Reddy *et al*, 1986; Sharma *et al*, 1989). Peroxisome proliferation of similar magnitude has not been observed in other cell types. The mRNA levels of the three genes encoding the peroxisomal β-oxidation system enzymes increased greater than 20-fold in the livers of rats treated with peroxisome proliferators, whereas in extrahepatic tissues the increases in mRNA levels were only 2- to 4-fold in kidney, small intestine and heart, and showed no significant change in nine other tissues studied (Nemali *et al*, 1988). The results correlate well with the levels of peroxisomal β-oxidation activity and immunologically quantitated proteins in various tissues (Nemali *et al*, 1988). This type of study suggests that peroxisome proliferators affect areas only in which they are absorbed or metabolized. It has also been observed that peroxisome proliferators induce tumours only in organs in which peroxisome proliferation occurs (Reddy, 1990). It is interesting that peroxisomal proliferation has been demonstrated in normal hepatocytes transplanted into extrahepatic tissue, which suggests that liver cells possess a specific mechanism to recognize these compounds (Reddy *et al*, 1984). A peroxisome-proliferator binding protein has been identified in the cytosolic fraction of rat hepatocytes (Lalwani *et al*, 1983). This observation has led to speculation that the binding of peroxisome proliferators by a specific receptor may mediate the induction of peroxisomes

(Lalwani *et al*, 1983). The peroxisome proliferator-binding protein has affinity for clofibric acid, ciprofibrate and nafenopin and was identified as a 70 kDa protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Lalwani *et al*, 1983; Alvares *et al*, 1990). The amino acid sequence of this protein exhibits homology to a constitutively expressed member of the rat heat shock protein 70 (HSP70) family, but the significance of this is unknown (Alvares *et al*, 1990). It is unlikely that this protein is a specific receptor involved in peroxisome-proliferator-induced gene expression because it is abundant in almost all organs of the rat (Alvares *et al*, 1990). It has been postulated that this HSP may be associated with the specific receptor (Alvares *et al*, 1990).

Another experimental approach has been used to identify a second potential peroxisome proliferator binding protein. The ability of these compounds to modulate specific gene transcription suggested to Issemann and Green (1990) that peroxisome proliferators could act by a mechanism similar to that of steroid hormones. Using homology to conserved regions of known steroid hormone receptors Issemann and Green (1990) identified a novel member of the steroid hormone receptor superfamily that is activated by peroxisomal proliferators. This receptor is activated by a group of chemically diverse peroxisome proliferators (clofibric acid, MEHP, Wy-14,643, nafenopin) and is thought to directly mediate the effects of

these chemicals (Issemann and Green, 1990). The physiological role of the putative natural ligand of this receptor is unknown; however, it may play a role in regulating fatty acid or cholesterol metabolism by affecting peroxisomal metabolic pathways (Issemann and Green, 1990). Understanding the function of this receptor will help to determine the basis for the peroxisomal proliferative, hypolipidemic and carcinogenic actions of these compounds.

It has been suggested that peroxisome proliferators constitute a novel class of chemical carcinogens (Reddy *et al*, 1980). Peroxisome proliferators do not appear to be directly genotoxic as they are non-mutagenic in the Ames test (Reddy *et al*, 1982) and they do not bind covalently to hepatic DNA after administration *in vivo* (von Däniken *et al*, 1984). It has been suggested that the carcinogenicity of these agents is due to their ability to induce oxidative DNA damage in the liver due to an imbalance in the production and degradation of peroxisomal $H_2O_2$ (Tomaszewski *et al*, 1986). The administration of peroxisome proliferators produces a marked increase in the enzymes of the peroxisomal $H_2O_2$-generating ß-oxidation pathway, while catalase levels are only slightly increased (Furuta *et al*, 1982; Reddy *et al*, 1986) leading to an overall increase in the level of $H_2O_2$ in hepatocytes (Tomaszewski *et al*, 1986). Oxidative damage may occur directly by $H_2O_2$ or by other reactive oxygen species (*e.g.* hydroxyl radicals) which may damage intracellular membranes and DNA. Kasai *et al*, (1989)

have shown that 8-hydroxydeoxyguanosine levels in rat liver DNA are increased upon chronic exposure to the peroxisome proliferator, ciprofibrate. This adduct is a result of damage to DNA caused by hydroxyl radicals (Kasai *et al*, 1989). There is evidence that in man (as well as in monkeys) hypolipidemic drugs do not elicit the peroxisome-proliferative response seen in rodents (Cohen and Grasso, 1981). Therefore one cannot extrapolate the findings of rodent hepatocarcinogenesis to humans.

The use of hypolipidemic drugs has facilitated the isolation of large quantities of peroxisomes and the study of peroxisomal functions. However, further experimentation is required to understand the mechanism of induction peroxisome proliferation and the basis for the variable responses observed among different species. In addition, much needs to be learned about how these chemicals act as hypolipidemic agents and rodent hepatocarcinogens.

## 1.6 The Peroxisomal Membrane

### 1.6.1 Properties of the Peroxisomal Membrane

Until recently, little was known of the properties of the peroxisomal membrane. Early studies on the osmotic behaviour of isolated peroxisomes established that the membrane is highly permeable (de Duve and Baudhuim, 1966). de Duve and Baudhuin (1966) determined that the peroxisomal

membrane is permeable to sucrose and that three peroxisomal oxidases (urate oxidase, D-amino acid oxidase, L-α-hydroxyacid oxidase) do not display latency, indicating their substrates readily permeate the membrane. More recent experiments have shown that isolated peroxisomes are permeable to small solutes, including sucrose and the cofactors for fatty acid oxidation ($NAD^+$, CoA, ATP and carnitine) (van Veldhoven *et al*, 1987). By reconstituting peroxisomal integral membrane polypeptides (IMPs) into phospholipid vesicles, van Veldhoven *et al*, (1987) concluded that the permeability properties of the peroxisomal membrane may be the result of a pore forming protein (molecular mass 22-28 kDa). The diameter of the channel (estimated to be 1.7 nm in rat liver peroxisomes) is large enough to allow the free diffusion of substrates, products and cofactors (van Veldhoven *et al*, 1987). Although these pores are thought to be responsible for the unusually high permeability of isolated peroxisomes, *in vivo* experiments suggest that microbodies are not leaky in intact cells. Visser *et al* (1981) showed by pulse-labeling experiments that the glycolytic intermediates of the glycosome of *Trypanosomes* do not readily mix with those in the cytosol. In addition, a $^{31}P$ NMR study concluded that the internal pH of peroxisomes of *H. polymorpha*, *Candida utilis* and *Trichosporon cutaneum* is 1.1 - 1.3 pH units below that of the cytosol (Nicolay *et al*, 1987). An ATPase has been localized to the peroxisomal membrane that may generate this pH gradient (Douma *et al*,

1987; del Valle *et al*, 1988); however, the functional role of this ATPase has not been determined. The existence of a pH gradient remains questionable, as it is difficult to imagine a pH gradient across a membrane which contains large pores. In addition, the enzymes of peroxisomes in animal tissues are known to have alkaline pH optima (Borst, 1989). However, further evidence for the existence of a proton gradient across the peroxisomal membrane comes from experiments showing that proton ionophores disrupt the *in vivo* import and assembly of alcohol oxidase in the methylotrophic yeast *Candida boidinii* (Bellion and Goodman, 1987). These results have been challenged, because the use of ionophores *in vivo* uncouples oxidative phosphorylation, thereby depleting cellular ATP, which may secondarily prevent import of peroxisomal proteins (Borst, 1989). Clearly, further experimentation is required to address this question. The different permeability behaviours of peroxisomes *in vitro* and *in vivo* may be reconciled if the permeability of the pores is regulated. For example, the pore may be selective *in vivo* but loses this selectivity mechanism during isolation procedures. The high permeability of the membrane of isolated peroxisomes casts doubt on the reliability of *in vitro* protein import experiments, assuming, of course, that efficient import is dependent on membrane integrity (Borst, 1989). This problem must be addressed by developing the methodology to isolate more intact peroxisomes.

1.6.2 Composition of the Peroxisomal Membrane

The rat liver peroxisomal membrane is approximately 6.8 nm thick and has a typical trilaminar appearance (Fujiki *et al*, 1982b). Studies of the composition of the peroxisomal membrane have contributed to our understanding of the organelle's biogenesis. These studies have focussed on comparisons of the peroxisomal membrane to those of other intracellular compartments. One might expect, for example, if the peroxisome is derived from the ER or another intracellular compartment, their respective membranes would exhibit similar compositions.

The phospholipid to protein ratio of rat liver peroxisomes is low compared to microsomes or mitochondria (0.086, 0.32 and 0.20 mg phospholipid/mg protein in peroxisomes, microsomes and mitochondria, respectively) (Donaldson *et al*, 1972). This is not unexpected, since the peroxisomal membrane constitutes only a small fraction of the total particle (Donaldson *et al*, 1972). The peroxisomal membrane has a smaller fraction of total organelle protein (12% of total) compared to microsomal membranes (53% of total) and mitochondrial membranes (21% of total) (Fujiki *et al*, 1982b). Donaldson *et al*, (1972) found that rat liver peroxisomal membranes are composed mainly of phosphatidyl choline (55.1%), phosphatidyl ethanolamine (16.0%) and phosphatidyl inositol (19.7%). The phospholipid composition is similar to that of the microsomal membrane, and both the

peroxisomal and microsomal membranes contain NADH-cytochrome c reductase and NADH-cytochrome $b_5$ reductase activities (Donaldson *et al*, 1972). Donaldson *et al*, (1972) suggested this was evidence that the peroxisomal membrane is derived from the ER. However, Fujiki *et al*, (1982b) also found the phospholipid composition of peroxisomal and microsomal membranes similar but, using SDS-PAGE analysis, showed that the polypeptide pattern of the peroxisomal membrane differs greatly from that of the ER. This result suggested that the ER does not play a role in peroxisome biogenesis (Fujiki *et al*, 1982b). In support of this, it has since been determined that NADH cytochrome $b_5$ reductase is synthesized on free polysomes (Borgese and Gaetani, 1980). Therefore its appearance in both peroxisomal and microsomal membranes is not evidence that the peroxisome forms by budding from the ER. Collectively, these studies have suggested that even though the phospholipid compositions are similar, the peroxisomal and ER membranes are independent, and the peroxisome does not form from the ER.

Rat liver peroxisomes contain the enzymes that catalyze the initial reactions in the formation of plasmalogens; however, they lack the other enzymes of phospholipid biosynthesis (Hajra and Bishop, 1982). These other enzymes are found mainly in the ER. Thus, the ER is probably the site of formation of phospholipids for the peroxisomal membrane, as it is thought to be for other

intracellular organelles (Hajra and Bishop, 1982; Vance, 1991). It is not known how these phospholipids are transferred to their final destinations; however, several mechanisms have been proposed for the interorganelle trafficking of lipids (Vance, 1991). It has been suggested that lipids may be transported intracellularly by carrier proteins (*e.g.* phospholipid exchange proteins), by vesicle-mediated transfer or as a result of physical contact between two membranes (Vance, 1991). It is also plausible that all of these mechanisms or some combination of these mechanisms occurs (Vance, 1991).

### 1.6.3 Biogenesis of the Integral Membrane Proteins

The biogenesis of the peroxisomal matrix proteins is well established. With few exceptions they are synthesized on free polysomes at their mature size and posttranslationally imported into existing peroxisomes. Until recently, only limited information was available concerning the polypeptides of the peroxisomal membrane. Studies of the peroxisomal membrane have been greatly facilitated by the development of a simple, rapid procedure for the isolation of membranes from purified peroxisomes (Fujiki *et al*, 1982a; 1982b).

Even after the sites of synthesis of many peroxisomal matrix proteins were determined, there remained some question as to the site of synthesis of the membrane proteins. The possibility existed that the peroxisomal membrane proteins

were synthesized on membrane-bound polysomes and co-translationally inserted into the ER, where they would be sorted and would direct the budding of an immature peroxisome (Goodman and Blobel, 1978; Köster *et al*, 1986). Subsequently, the peroxisomal matrix proteins, synthesized on free polysomes, could be posttranslationally imported via receptors in the peroxisomal membrane to form the mature organelle (Goodman and Blobel, 1978; Köster *et al*, 1986). The first piece of evidence against this hypothesis was the finding that a major IMP of rat peroxisomes (22 kDa) is synthesized on free polysomes without a precursor extension (Fujiki *et al*, 1984). Since then, it has been shown that the 22 kDa, 36 kDa and 69 kDa rat liver peroxisomal IMPs synthesized *in vitro* do not differ in size from their mature polypeptides (Köster *et al*, 1986) and that the 22 kDa and 69 kDa IMPs are synthesized on free polysomes (Suzuki *et al*, 1987). From these results it has been suggested that all peroxisomal proteins are synthesized on free polysomes and posttranslationally imported, as appears to be the case for mitochondrial and chloroplast proteins (Pugsley, 1989). However, although most proteins of the secretory pathway are synthesized on membrane-bound polysomes, there are a few exceptions, notably cytochrome $b_5$ (Rachubinski *et al*, 1980) and NADH-cytochrome $b_5$ reductase (Borgese and Gaetani, 1980). These exceptions warrant against making generalizations about peroxisomes based on the results of a few membrane proteins.

The mechanism by which polypeptides eventually become incorporated into the peroxisomal membrane is not known. By pulse-labeling experiments, Just and Hartl (1987) have detected the peroxisomal 69 kDa and 22 kDa IMPs transiently in the cytoplasm before import into the membrane. Their presence in the cytoplasm may be explained by association with water-soluble components or the result of folding of the polypeptides to expose hydrophilic regions (Just and Hartl, 1987). The requirements for import of the membrane proteins into the peroxisomal membrane may be dissected by future *in vitro* import studies. Recent isolation of the cDNAs encoding two rat liver peroxisomal IMPs (35 kDa IMP and 70 kDa IMP) will certainly facilitate these studies (Kamijo *et al*, 1990; Tsukamoto *et al*, 1991). The 70 kDa IMP has similarity to members of the ATP-binding protein family, which includes several proteins involved in bacterial transport systems and the protein that confers multi-drug resistance to mammalian tumor cells (P-glycoprotein) (Kamijo *et al*, 1990). It is suspected that this protein is involved in active transport across the peroxisomal membrane (Kamijo *et al*, 1990). The 35 kDa IMP restores the biogenesis of peroxisomes in a peroxisome-deficient mutant of CHO cells, and it has been speculated that it is a component of a putative signal receptor or of the transmembrane import machinery of peroxisomes (Tsukamoto *et al*, 1991).

Most of the major peroxisomal IMPs have been

identified primarily by SDS-PAGE and therefore are characterized by their molecular weight. Little is known of the biological functions of these proteins. So far, the peroxisomal membrane proteins characterized enzymatically (*e.g.* NADH-cytochrome c reductase, acyl-CoA synthase and dihydroxyacetone phosphate acyltransferase) are only minor constituents of the membrane and do not correspond to the IMPs identified according to size (Hartl and Just, 1987). Elucidation of the functions of some of the major peroxisomal IMPs awaits further experimentation.

## 1.7 Peroxisomal Disorders

For many years, the function of mammalian peroxisomes was not considered significant. Evidence that peroxisomes have a significant function in mammals is provided by the severity of clinical manifestations associated with human peroxisomal disorders (Moser, 1987). Studying peroxisomal disorders provides the opportunity to learn more about peroxisome formation and function. Peroxisomal disorders show a great deal of phenotypic and genotypic heterogeneity and have tentatively been divided into three classifications (Moser, 1986). The first group is referred to as generalized peroxisomal disorders. This group is characterized by a reduced number of peroxisomes and deficient activity of multiple peroxisomal enzymes. In this case the primary defect is thought to involve the biogenesis or maintenance of the

organelle (Moser, 1986). In the second group, the number of peroxisomes is normal but the activities of more than one peroxisomal enzyme are reduced. Group three is characterized by a defect in a single peroxisomal enzyme that does not otherwise affect the structure or function of the peroxisome (Moser, 1986; 1987). A list of peroxisomal disorders and their characteristics is shown in Table 1.7.1. This discussion will be limited to generalized peroxisomal disorders, in particular Zellweger syndrome, because it is the most extensively characterized and has contributed greatly to our understanding of peroxisome biogenesis in recent years.

Upon examination of electron micrographs of liver biopsies from infants with Zellweger syndrome it was noticed that there were no morphologically detectable peroxisomes (Goldfischer *et al*, 1973). The significance of this finding was not appreciated until the functions of mammalian peroxisomes were defined. Cells from Zellweger patients were shown to have deficient activities in the oxidation of long-chain fatty acids, synthesis of plasmalogens and synthesis of bile acids (Moser and Goldfischer, 1985; Moser, 1987). Deficiencies in these pathways lead to elevated levels of long-chain fatty acids, accumulation of bile acid precursors and markedly reduced plasmalogen levels in the tissues and fluids of Zellweger patients (Moser and Goldfischer, 1985). Early biochemical analysis illustrated that some peroxisomal matrix enzymes (e.*g*. catalase, L-α-hydroxyacid oxidase and D-

Table 1.7.1 Peroxisomal Disorders

| | | |
|---|---|---|
| **Group I:** | **Deficient activities of multiple peroxisomal enzymes and decreased peroxisomal number** | |
| Zellweger (cerebrohepatorenal) syndrome (ZS) | - | peroxisomes are absent by morphological analysis |
| | - | decreased β-oxidation of fatty acids, bile acid synthesis and plasmalogen biosynthesis |
| | - | defect in peroxisomal import machinery |
| Neonatal adrenoleukodystrophy (NALD) | - | clinically and biochemically similar to ZS; however, clinical manifestations are often not as severe and develop slightly later in life than ZS. |
| Infantile Refsum's syndrome | - | similar to NALD |
| hyperpipecolic acidemia | - | similar to but less severe than ZS, initially characterized by high levels of serum and urinary pipecolic acid |
| Rhizomelic chondrodysplasia punctata | - | characterized by a deficiency of plasmalogen biosynthesis, phytanate catabolism and a partial deficiency of 3-ketoacyl-CoA-thiolase activity |
| **Group II:** | **Decreased activities of multiple peroxisomal enzymes and normal peroxisomal number** | |
| Pseudo-Zellweger syndrome | - | clinical symptoms similar to ZS but abundant peroxisomes which stain for catalase |
| **Group III:** | **Decreased activity of a single peroxisomal enzyme** | |
| X-linked adrenoleukodystrophy | - | deficient very long-chain fatty acyl-CoA ligase (lignocerol CoA ligase) |
| | - | defective peroxisomal oxidation of very long-chain fatty acids |
| Refsum's disease | - | deficient phytanic acid oxidase |
| | - | inability to oxidase phytanic acid (a C-20 fatty acid of dietary origin) |
| Acatalasemia | - | deficient peroxisomal catalase |
| Hyperoxaluria I | - | deficient peroxisomal alanine:glyoxylate aminotransferase |
| | - | overproduction of oxalate |

Table constructed from Moser and Goldfischer (1985); Moser (1986; 1987); Talwar and Swaiman (1987); Zellweger (1987).

amino acid oxidase) are synthesized in patients with Zellweger syndrome but are found in the cytosol rather than in the organellar fraction of cells (Moser and Goldfischer, 1985; Moser, 1987). In contrast, certain enzymes of the peroxisomal membrane, notably dihydroxyacetone phosphate acyltransferase, are markedly diminished (Moser and Goldfischer, 1985).

These results led to the speculation that the underlying defect in Zellweger syndrome is impaired biogenesis of the peroxisomal membrane (Moser and Goldfischer, 1985). The autosomal recessive mode of inheritance in Zellweger syndrome suggests that the defect involves a single gene. Therefore it was postulated that this mutation affects the formation of protein(s) that generate the peroxisomal membrane (Moser and Goldfischer, 1985). This original hypothesis has been challenged by a number of findings. Lazarow *et al*, (1986) found that the peroxisomal 22 kDa IMP is present in normal amounts in liver cells from a Zellweger patient and is integral to a membrane. It was therefore suggested that peroxisomal membranes are assembled in Zellweger syndrome but may be defective for the import of matrix proteins (Lazarow *et al*, 1986). This result was substantiated by immunofluorescence microscopy experiments using antibodies against the peroxisomal membrane proteins (Santos *et al*, 1988b). In Zellweger fibroblasts, the peroxisomal membrane proteins are located in empty membrane enclosed vesicles referred to as peroxisomal 'ghosts' (Santos *et al*, 1988a; 1988b).

Collectively, these results suggest that the primary defect in Zellweger syndrome is not in the assembly of the peroxisomal membrane but is in an inability to import proteins into the peroxisome matrix (Santos *et al*, 1988a; 1988b; Suzuki *et al*, 1989). As a result, newly synthesized proteins remain in the cytosol, where some mature and remain stable (*e.g.* catalase), while others are degraded (Lazarow *et al*, 1986). This explains the varying levels of peroxisomal enzymes that have been reported in Zellweger cells. It has also been suggested that the empty peroxisomal vesicles, lacking their characteristic granular matrix, are not recognizable morphologically, explaining earlier findings (Lazarow *et al*, 1986).

These results have led to the realization that studying Zellweger syndrome and other generalized peroxisomal disorders will aid in understanding the process of protein import into peroxisomes. Complementation experiments involving the fusion of fibroblasts from patients afflicted with a variety of peroxisomal disorders have led to the identification of at least six separate complementation groups (Brul *et al*, 1988a; Roscher *et al*, 1989; McGuinness *et al*, 1990). In each study, fibroblasts from two cell lines deficient in peroxisomal metabolic processes are fused and examined for the restoration of functional peroxisomes. Restoration of peroxisomal activity can only occur if one parental cell line provides the gene product defective in the

other (McGuinness *et al*, 1990). The existence of six complementation groups indicates that at least six gene products are required for the biogenesis of peroxisomes. Interestingly, the kinetics of complementation have shown that in some pairs of complementing cell lines, complementation is very rapid and unaffected by the addition of cycloheximide, an inhibitor of protein synthesis (Brul *et al*, 1988b). This result illustrates that in some cases pre-existing components can be redistributed to reconstruct the import machinery (Brul *et al*, 1988b). Another interesting observation from these studies is that the currently used clinical categories do not represent distinct genotypes (Roscher *et al*, 1989). This suggests that different mutant genes may result in a similar phenotype and that one defective gene may lead to variant phenotypes (Roscher *et al*, 1989).

Although tissues and fibroblast cell lines from Zellweger syndrome patients have been useful for biochemical analyses, their use in genetic studies is limited because primary cell lines do not proliferate indefinitely in culture and are difficult to grow clonally (Zoeller *et al*, 1989). Because of the advantages of other cell systems, several of these have been used to create mutants whose characteristics are phenotypically similar to those of cells from patients with Zellweger syndrome. These peroxisome assembly (*pas*) mutants have been created in the yeast *S. cerevisiae* (Erdmann *et al*, 1989) as well as in Chinese hamster ovary (CHO) cells

(Zoeller and Raetz, 1986; Zoeller *et al*, 1989). The existence of these *pas* mutants should greatly facilitate the molecular analyses of the various processes of import of peroxisomal proteins. By complementation of these mutants with DNA, two recombinants have been identified that are required for the biosynthesis of peroxisomes. *PAS1* is a yeast gene required for peroxisome biogenesis in *S. cerevisiae* (Erdmann *et al*, 1991). This gene encodes a hydrophilic polypeptide with an approximate molecular mass of 117 kDa, possessing two putative ATP-binding sites (Erdmann *et al*, 1991). It has been suggested that the *PAS1* gene product is a soluble component of the import machinery; however, further characterization is required in order to delineate the role of this protein (Erdmann *et al*, 1991). In addition, Tsukamoto *et al*, (1991) have identified a rat liver cDNA that restores the biogenesis of peroxisomes in a peroxisome-deficient CHO mutant cell line. This cDNA encodes a 35 kDa peroxisomal membrane protein termed peroxisome assembly factor 1 (PAF-1) (Tsukamoto *et al*, 1991). It has been suggested that this protein may be a component of a putative signal receptor or of the transmembrane import machinery of peroxisomes; however, its role has not been determined experimentally (Tsukamoto *et al*, 1991).

## 1.8 The Project

Two distinct aspects of rat liver peroxisomes were investigated in this work. The first part of the project

involved the characterization of the rat liver peroxisomal membrane proteins. The second part included the selection of a cDNA recombinant encoding rat liver peroxisomal 3-ketoacyl-CoA thiolase with subsequent studies on the targeting of the protein to peroxisomes. The details of each project are introduced at the onset of the appropriate sections of the Results and Discussion and will therefore only be briefly outlined below.

At the onset of this project, little was known about the peroxisomal membrane proteins. The objective of this work was to characterize the integral membrane proteins of rat liver peroxisomes in an effort to understand further the biogenesis of this organelle. An antiserum was raised against purified peroxisomal membranes which reacted with six IMPs. This antiserum was used to investigate the co-localization of these IMPs to other subcellular organelles, the response of these IMPs to treatment with the hypolipidemic drug clofibrate and the cellular site of synthesis of several of these IMPs.

The second part of this thesis involved the selection and characterization of a cDNA recombinant for peroxisomal 3-ketoacyl-CoA thiolase. This particular protein is unique in rat liver peroxisomes in that it is synthesized as a larger precursor with an amino-terminal extension, which is cleaved to yield the mature protein. The main objective of this section was to determine if the cleavable presequence plays a role in targeting this protein to peroxisomes. To facilitate

these studies, a cDNA recombinant encoding peroxisomal 3-ketoacyl-CoA thiolase was selected and characterized. This cDNA recombinant was then used in an established *in vivo* targeting system to determine the role of the precursor extension in peroxisomal targeting.

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemicals and Reagents

The following is a list of critical reagents and the companies from which they were purchased. All chemicals and reagents were of the highest quality available.

| | |
|---|---|
| ampicillin | Sigma Chemical Company |
| antipain | Sigma Chemical Company |
| bacto-agar | Difco Laboratories |
| Bio-Rad protein assay dye reagent | Bio-Rad Laboratories (Canada) Ltd. |
| 5-bromo-4-chloro-3-indolyl phosphate (BCIP) | Promega Corporation |
| chymostatin | Sigma Chemical Company |
| clofibrate | Sigma Chemical Company |
| Coomassie Brilliant Blue (R-250) | Bethesda Research Laboratories Inc.(BRL) |
| cytochrome-c (horse heart) | Sigma Chemical Company |
| deoxyribonucleotides (dATP, dGTP, dCTP, dTTP) | Pharmacia (Canada) Inc. |
| Freund's complete adjuvant | Sigma Chemical Company |
| GTG agarose | FMC BioProducts |
| guanidine thiocyanate | Fluka Chemical Corp. |
| hemoglobin (bovine blood) | Sigma Chemical Company |
| hydrogen peroxide (30%) | Sigma Chemical Company |
| isopropyl ß-D-thiogalactoside (IPTG) | Gibco/BRL Canada |
| leupeptin | Sigma Chemical Company |
| low melting point agarose | Gibco/BRL Canada |
| molecular weight standards: | |
| (i) 1 kbp DNA ladder (75-12,216 bp) | Gibco/BRL Canada |
| (ii) Dalton Mark VII-L molecular weight markers for SDS-PAGE (14,000-66,000 Da) | Sigma Chemical Company |
| (iii) phosphorylase b (rabbit muscle; 97,400 Da) | Sigma Chemical Company |
| (iv) ß-galactosidase (*E. coli*; 116,000 Da) | Sigma Chemical Company |
| nitro blue tetrazolium (NBT) | Promega Corporation |
| nitrocellulose (pore size-0.45µm) | Schleicher and Schuell Inc. |

| | |
|---|---|
| o-nitrophenyl acetate | Sigma Chemical Company |
| p-nitrophenyl-N-acetyl-ß-D-glucosaminide | Sigma Chemical Company |
| Nonidet P-40 (NP-40) | Sigma Chemical Company |
| oligo(dT)$_{12-18}$ | Collaborative Research Inc. |
| oligo(dT) cellulose, Type 3 | Collaborative Research Inc. |
| ovalbumin | Miles Laboratory (Pty) Ltd. |
| PANSORBIN *Staphylococcus aureus* cells (inactivated) | Calbiochem Corp. |
| pepstatin A | Sigma Chemical Company |
| RNasin RNase inhibitor | Progmega Corporation |
| Sephadex G-50 (medium) | Pharmacia (Canada) Inc. |
| sodium carbonate | Sigma Chemical Company |
| sodium hydrosulfite | Sigma Chemical Company |
| titanium oxysulfate hydrate | Aldrich Chemical Company Inc. |
| Triton WR-1339 (tyloxapol) | Sigma Chemical Company |
| Triton X-100 | Sigma Chemical Company |
| tRNA (calf liver) | Boehringer Mannheim |
| Tween 20 (polyoxyethylene sorbitan monolaurate) | Sigma Chemical Company |
| X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) | Gibco/BRL Canada |

### 2.1.2 Radiochemicals

| | |
|---|---|
| $^{125}$I-protein A (>30 mCi/mg total protein A, 0.1 Ci/µl) | Amersham Canada Ltd. |
| [γ-$^{32}$P]ATP (3,000 Ci/mmol, 10 µCi/µl) | Amersham; Dupont/NEN Canada Inc. |
| [α-$^{32}$P]dATP (3,000 Ci/mmol, 10 µCi/µl) | Amersham; ICN Biomedicals Inc. |
| L-[$^{35}$S]-methionine (1151 Ci/mmol 10 µCi/µl) | Dupont/ NEN Canada Inc. |

### 2.1.3 Enzymes

All enzymes were used according to the manufacturer's product data sheets unless otherwise specified in the methods section.

| | |
|---|---|
| DNA ligase (*E. coli*) | Amersham Canada Ltd.; Pharmacia (Canada) Inc. |

| | |
|---|---|
| DNA ligase (T4) | Gibco/BRL; New England Biolabs; Promega Corp. |
| DNA polymerase I Klenow fragment (*E. coli*) | New England Biolabs, Inc. |
| DNA polymerase, modified T7 (Sequenase) | United States Biochemical Corp. |
| DNA polymerase (*Thermus aquaticus*) (Taq polymerase) | Perkin-Elmer-Cetus |
| DNase I (bovine pancreas) | Sigma Chemical Company |
| exonuclease III (*E. coli*) | Amersham Canada Ltd.; Gibco/BRL Canada |
| polynucleotide kinase (T4) | Pharmacia (Canada) Inc. |
| restriction endonucleases | Gibco/BRL; New England Biolabs; Pharmacia |
| reverse transcriptase (avian myeloblastosis virus) | Pharmacia (Canada) Inc. |
| RNase I "A" (bovine pancreas) | Pharmacia (Canada) Inc. |
| terminal deoxynucleotidyl transferase (TdT) (calf thymus) | Gibco/BRL |

### 2.1.4 Antisera

Mouse anti-rabbit IgG (Fc) alkaline phosphatase conjugate and mouse monoclonal anti-ß-galactosidase were purchased from Promega Corporation. Rabbit anti-rat cytochrome P-450 PB-1 and mouse monoclonal anti-rat ribophorin were gifts from Dr. Gert Kreibach, Department of Cell Biology, New York University, New York, NY. Goat anti-mouse IgG alkaline phosphatase conjugate was a gift from Dr. D.W. Andrews, Department of Biochemistry, McMaster University. The rabbit anti-bovine catalase serum used for immunofluorescence

microscopy was a gift from Dr. André Schram, Laboratory of Biochemistry, University of Amsterdam, The Netherlands. The rabbit anti-*Neurospora crassa* porin serum was a gift from Dr. Carmen A. Manella, Department of Biological Sciences, State University of New York at Albany, Albany, NY. The CAT2 mouse monoclonal anti-chloramphenicol acetyltransferase antibodies were a gift from Dr. C. Gorman, Genentech, San Francisco, CA. The anti-SKL antibodies were supplied by Dr. S.J. Gould, Department of Biology, University of Calfornia at San Diego, La Jolla, CA. Rabbit anti-rat thiolase and rabbit anti-rat peroxisomal IMPs were raised in accordance with the procedures outlined in Materials and Methods, section 2.5.1.

### 2.1.5. cDNA Library, Cloning Vectors and Host Bacterial Strains

The λgt11 rat liver cDNA library and host *E. coli* Y1090 and Y1089 cells were obtained from Clontech Laboratories, Inc. To facilitate sequencing and other DNA manipulations cDNA inserts were subcloned into the plasmid vectors pGEM-5Zf(+) or pGEM-7Zf(+) (Promega Corporation) or pUC118 (Vieira and Messing, 1987), which was obtained from Dr. A.B. Futcher, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (original source was Dr. J. Vieira, Department of Biochemistry, University of Minnesota). These plasmids were transformed into *E. coli* DH5α competent cells purchased from Gibco/BRL. Growth media for *E. coli* cells was Luria broth

(LB) [1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 1% (w/v) NaCl, pH 7.5] (Maniatis *et al*, 1982), containing ampicillin (100 μg/ml) for transformed cells.

### 2.1.6 Vectors and Cell Lines Used for Transfections and Immunofluorescence

The cell lines used for transfections and immunofluorescence were CV-1 monkey kidney cells or CV-H Px110 cells. The CV-H Px110 cell line was obtained by hygromycin selection of CV-1 cells co-transfected with pSV2-CATC-PMP20 (Gould *et al*, 1990a) and pTK-Hyg (Sugden *et al*, 1985). The CV-H Px110 cell line expresses the peroxisomally located CAT-PMP20 fusion protein in about 50% of the cells. The expression vector pRSV-An was used for the expression of the thiolase gene constructs. The plasmid pRSV-An was derived from pRSV-CAT (Gorman *et al*, 1982a) in which the CAT containing *Hind*III-*Bam*HI fragment was replaced with sequences derived from the *Bcl*I-*Bam*HI SV40 fragment (position 2770-2533) which contains the early region polyadenylation signal (An). The expression vector pSV2-Not-leader-CAT was constructed from pSV2-CAT (Gorman *et al*, 1982b) with a *Not*I site and other restriction sites from the pUC8 polylinker immediately upstream of the CAT translational initiation site. The expression of CAT in this construct is under the control of the SV40 early region promoter. These cell lines and vectors were obtained or constructed in the laboratory of Dr. S.

Subramani, Department of Biology, University of California at San Diego. The plasmid pTZ-19U used in some of the DNA manipulations was obtained from Pharmacia.

### 2.1.7 Oligodeoxyribonucleotides

The following oligodeoxyribonucleotides were used for primer extension and for analysis of mRNAs encoding rat liver peroxisomal thiolase or the 50 kDa IMP (Materials and Methods, section 2.18 and 2.19). Oligodeoxyribonucleotides were synthesized using an Applied Biosystems 381A Automated DNA Synthesizer by the Central Facility of the Institute of Molecular Biology and Biotechnology, McMaster University.

Table 2.1.1 Oligodeoxyribonucleotides

| Primer | oligodeoxyribonucleotide sequence (annealing position) | purpose |
|---|---|---|
| AB704 | 5′-CAATGCGCGCCATGACTG-3′ (340-323) | RT-primer, used to direct primer extension of mRNA encoding thiolase 2. |
| AB616 | 5′-TCCTGGCTCAAGTACGTTA-3′ (315-297) | AMP-primer, used for PCR amplification of cDNA encoding thiolase 2, and northern and slot blot analyses. |
| AB755 | 5′-GGAAGGTAGCGGAGC-3′ (121-107) | Probe for colony hybridization and Southern blot analyses of the cDNA encoding thiolase 2. |
| AB703 | 5′-GACTCGAGTCGACATCGA$(T)_{17}$ | $(dT)_{17}$-adaptor, used to direct second-strand cDNA synthesis. |
| AB705 | 5′-GACTCGAGTCGACATCG-3′ | Adaptor, used for the PCR amplification of cDNA. |

| Primer | oligodeoxyribonucleotide sequence (annealing position) | purpose |
|---|---|---|
| AB615 | 5′-CCCGGGCTGAAGCACATTG-3′ (285-267) | Probe for thiolase 1 mRNA, used for northern and slot blot analyses. |
| AB792 | 5′-TCCACTGAAGCCAGTCTGA-3′ (204-186) | Used to direct primer extension of mRNA encoding the 50 kDa IMP. |
| AB496 | 5′-GGGTGGACGGTCCCCTGGAG-3′ (158-139) | Used for PCR amplification of cDNA encoding the 50 kDa IMP. |
| AB497 | 5′-GGTTCATGTATGGAG-3′ (79-65) | Used for colony hybridization and Southern blot analysis of cDNA and northern blot analyses of mRNA encoding the 50 kDa IMP. |

The M13 forward (universal) sequencing primer and the M13 reverse sequencing primer, used for dideoxy sequencing from pGEM plasmid vectors (section 2.15), were purchased from United States Biochemical Corp. or Pharmacia (Canada) Inc.

pUC/M13 forward sequencing primer 5′-GTAAAACGACGGCCAGT-3′

pUC/M13 reverse sequencing primer 5′-CAGGAAACAGCTATGAC-3′

## 2.2 Organelle Isolations and Marker Enzyme Analyses

### 2.2.1 Isolation of Peroxisomes, Mitochondria and Lysosomes from Rat Liver

Peroxisomes, mitochondria and lysosomes were isolated from the livers of female Sprague-Dawley rats by differential centrifugation followed by equilibrium density centrifugation as described by Leighton *et al* (1968) and modified by Alexson *et al* (1985). Initially, the rats were injected

intraperitoneally with Triton WR-1339 (85 mg per 100 g body weight) 3.5 days before sacrifice. The rats were sacrificed by decapitation, and the livers were excised and immersed in a tared beaker containing ice-cold 0.25 M sucrose in imidazole buffer [3 mM imidazole-HCl (pH 7.2), 0.1% ethanol (v/v) and 1 mM EDTA]. The livers were weighed, minced finely and homogenized in sucrose-imidazole buffer to give a 20% (w/v) homogenate. Homogenization was performed using a lab stirrer (model 4376-00; Cole-Parmer Instrument Company) in a smooth-walled glass grinding vessel fitted with a teflon pestle (Thomas Scientific) driven at about 1,000 rpm for one stroke. The homogenate was centrifuged at 2,440 x $g_{av}$ for 10 minutes at 4°C in a Sorvall HB4 rotor (Dupont/New England Nuclear Canada Inc.). The post-nuclear supernatant was removed and the pellet was rehomogenized [20% (w/v)] in sucrose-imidazole buffer and centrifuged as before. The combined pellets were rehomogenized [20% (w/v)] and kept for marker enzyme analyses. This fraction contained unbroken cells, connective tissues, erythrocytes, gross cell debris, cell membranes, most of the nuclei and a large proportion of the mitochondria and will be referred to as a heavy mitochondrial fraction (v) (Leighton *et al*, 1968). The combined supernatants were mixed and centrifuged at 17,100 x $g_{av}$ for 20 minutes at 4°C in a Sorvall HB4 rotor. The supernatant and pink fluffy layer were removed and the pellet was gently worked into a smooth paste with a glass rod, resuspended in sucrose-imidazole buffer (0.25 of

the previous volume) and recentrifuged. The combined supernatants which included the microsomal and soluble fractions were saved for marker enzyme analyses and will be referred to as a crude microsomal fraction ($\psi$) (Leighton *et al*, 1968). The pellet, which contained primarily mitochondria, peroxisomes and lysosomes [referred to as the light mitochondria fraction ($\lambda$ fraction)], was resuspended in a minimal volume of sucrose-imidazole buffer with a glass rod. Subfractionation of the $\lambda$ fraction was carried out by isopycnic centrifugation in a Beckman Type VTi50 rotor (Beckman Instruments, Inc.) . A linear sucrose density gradient (total volume of 32 ml) between 1.15 g/cm$^3$ and 1.27 g/cm$^3$ was overlaid on a 2.2 ml sucrose cushion of 1.32 g/cm$^3$ (all sucrose solutions were made in imidazole buffer). The $\lambda$ fraction (2.5 ml) was layered on the top of the gradient, and the sample was overlaid with sucrose-imidazole buffer to fill the Beckman quick-seal tube. Centrifugation was carried out at 4°C at 200,000 x $g_{av}$ to yield an $\int\omega^2dt$ value of 6.66 x $10^{10}$ $s^{-1}$ in order to obtain the same effective centrifugation used by Leighton *et al* (1968). The Beckman L8-M ultracentrifuge (Beckman Instruments Inc.) was equipped with an automatic rate controller for slow acceleration and slow deceleration below 3,000 rpm. Twenty 2 ml fractions were collected from the bottom of the gradient with a polystaltic pump (Buchler Instruments). The purity of all subcellular fractions was determined by marker enzyme analyses: catalase for

peroxisomes, cytochrome c oxidase for mitochondria, N-acetyl-ß-glucosaminidase for lysosomes and esterase for microsomes. Where indicated, the rats were treated for 10 days prior to organelle isolation with 0.5% (w/w) clofibrate in Purina rat chow to induce hepatic peroxisomal proliferation (Rachubinski *et al*, 1984). The clofibrate rat chow was prepared by mixing 0.5 g clofibrate, dissolved in 65 ml ether, per 100 g powdered Purina rat chow. The mixture was formed into pellets and dried overnight in a 60°C oven.

### 2.2.2 Preparation of Rat Liver Microsomes

A highly purified rat liver microsomal fraction was prepared from clofibrate-treated Sprague-Dawley rats by the procedure of Bergeron (1979) as modified by Paiement and Bergeron (1983). The rats were sacrificed by decapitation, and the livers were excised and homogenized to give a 20% (w/v) homogenate. Homogenization was performed in 0.25 M sucrose, containing 4 mM imidazole (pH 7.4) using a Cole-Parmer lab stirrer in a glass grinding vessel fitted with a teflon pestle (Thomas Scientific) driven at 1,000 rpm for six strokes. Initially, the homogenates were centrifuged at 8,700 x $g_{av}$ for 13 minutes in a Servall SS-34 rotor (Dupont/ NEN Canada Inc.) to yield a combined nuclear/mitochondrial pellet. The supernatant was further centrifuged at 40,000 x $g_{av}$ for 6 minutes, 40 seconds in the Beckman Type 65 rotor (Beckman Instruments Inc.). Microsomes were pelleted from the

supernatant by centrifugation at 100,000 x $g_{av}$ for one hour in the Beckman Type 65 rotor and then resuspended in sucrose to give a final sucrose concentration of 1.38 M. This suspension was underlaid beneath a discontinuous gradient comprised of 1.0 M, 0.86 M and 0.25 M sucrose (0.95 ml each) and centrifuged in a Beckman SW 50.1 rotor (Beckman Instruments Inc.) at 120,000 x $g_{av}$ for 150 minutes. The rough microsomal pellet was resuspended in 0.25 M sucrose containing 4 mM imidazole buffer (pH 7.4) and assayed for enzymatic activities and protein content.

### 2.2.3 Catalase Assay

The subcellular fractions (50 $\mu$l) were incubated with 1% (w/v) Triton X-100 and a substrate solution [20 mM imidazole buffer (pH 7.0), 0.1% bovine serum albumin and 0.01% $H_2O_2$] in a final volume of 1 ml for 5 minutes at 0°C. The reaction was stopped by the addition of 1 ml of a saturated solution of $TiOSO_4$ (titanium (IV) oxysulfate) in 2 N $H_2SO_4$ and the remaining $H_2O_2$ was determined colorimetrically as a yellow complex (peroxy-titanium sulfate), at 410 nm (Baudhuin *et al*, 1964).

### 2.2.4 Cytochrome c Oxidase Assay

Horse heart cytochrome c was dissolved in 0.03 M ammonium acetate (pH 7.4) to give a final concentration of 0.38 mg/ml. To a 3 ml spectrophotometer cuvette, 2.5 ml of

the cytochrome c solution, 0.25 ml of 10% (w/v) Triton X-100 and a spatula tip of sodium hydrosulfite (to reduce cytochrome c) were added. The solution was mixed vigorously to dissolve the hydrosulfite. The fractions were added and the rate of oxidation of cytochrome c by cytochrome c oxidase was measured on a chart recorder as a decrease in absorbance at 550 nm (Cooperstein and Lazarow, 1951).

### 2.2.5 Esterase Assay

The subcellular fractions (50 µl) were added to a freshly made solution containing 20 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 0.1% (w/v) Triton X-100 and 0.652 mg/ml of o-nitrophenyl acetate in a 1.0 ml reaction volume. The production of o-nitrophenol from o-nitrophenyl acetate was monitored at 420 nm (Beaufay *et al*, 1974a).

### 2.2.6 N-Acetyl-ß-Glucosaminidase Assay

N-acetyl-ß-glucosaminidase activity was measured as described by Findlay *et al* (1958). The fractions were incubated for 1 hour at 37°C in 0.1 M citrate buffer (pH 5.0), 0.1% (w/v) Triton X-100 and 5 mM p-nitrophenyl-N-acetyl ß-D-glucosaminide in a final volume of 0.5 ml. The reaction was stopped by the addition of 3 ml of glycine buffer (0.133 M glycine, 0.083 M $Na_2CO_3$, 0.067 M NaCl, pH 10.5) and the liberated p-nitrophenol was measured spectrophotometrically at 400 nm.

## 2.3 Analyses of Proteins

### 2.3.1 Bradford Method of Protein Determination

Protein content of samples was determined by the Bradford method (Bradford, 1976) using ovalbumin as a standard. Protein solutions containing 1 to 20 μg protein in a final volume of 0.1 ml were mixed with 1.0 ml of diluted Bio-Rad protein dye reagent [0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid] (Bio-Rad Laboratories). The binding of Coomassie Brilliant Blue G-250 to protein causes a shift in absorption maximum of the dye from 465 to 595 nm and the increase in absorption at 595 nm was monitored spectrophotometrically.

### 2.3.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) and modified by Fujiki *et al* (1984). Separating gels were cast as either 14 cm x 12 cm or 14 cm x 28 cm, 7-15% gradient acrylamide using the Hoefer vertical slab gel unit Model SE 400 (Hoefer Scientific Instruments). The gradient gels were poured with a Hoefer gradient maker (Model SG 15 or SG 30). Both components of the separating gel, 7% and 15% acrylamide [(30:0.8) acrylamide:N,N'-methylene-bis-acrylamide (w/w)], contained 0.37 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.05% (v/v) N,N,N',N'-

tetramethylethylenediamine (TEMED) and 0.2% (w/v) ammonium persulfate. In addition, the 15% acrylamide component contained 15% (w/v) sucrose to increase its density. The stacking gels consisted of 3% (w/v) acrylamide in 60 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulfate. Protein samples were prepared by boiling for 5 minutes in SDS-PAGE sample buffer [0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% sucrose, 0.01 M dithiothreitol (DTT) and 0.001% bromophenol blue]. Electrophoresis was carried out until the tracking dye reached the bottom of the separating gel, at 50 V overnight or 250 V for approximately 4 hours, in electrophoresis running buffer [0.4 M glycine, 50 mM Tris base, 0.1% (w/v) SDS, pH 8.8]. The gels were stained for 1 hour with 0.1% Coomassie Brilliant Blue (R-250) in 10% (v/v) acetic acid and 35% (v/v) methanol and diffusion-destained by repeated washing in 10% (v/v) acetic acid and 35% (v/v) methanol. Gels were dried under vacuum at 80°C using a Bio-Rad gel dryer (Model 483).

### 2.3.3 Preparative SDS-PAGE and Electroelution of Polypeptides From Acrylamide Gels

To purify large quantities of a polypeptide, the components of a protein mixture were separated by SDS-PAGE and the polypeptide of interest was electroeluted from the acrylamide gels (Fujiki *et al*, 1984). The SDS-polyacrylamide gels were set up exactly as described in section 2.3.2 except

that the dimensions of the separating gels were 14 cm x 28 cm and the stacking gel was cast with only two wells. One well (1 cm wide), was used for $M_r$ markers and the other well (11.5 cm wide) was used for the protein sample. Samples usually contained 0.5 mg to 1.5 mg of total protein (not less than 5-10% being the protein of interest). Following electrophoresis, the gels were stained and destained as usual, and the protein bands were excised with a razor blade. Electroelution of protein from the gel slice was performed in a Bio-Rad isoelectric focussing (IEF) chamber (Tube Cell, model 155). A 10 ml serological pipette, blocked at the narrow end with siliconized glass wool, was fitted in place of an IEF tube. The top and bottom of the IEF chamber were filled with electrophoresis running buffer [0.4 M glycine, 50 mM Tris base, 0.1% (w/v) SDS, pH 8.8] and the gel slice was placed into the 10 ml pipette. The protein was eluted from the gel slice into a sealed dialysis bag which was attached to the end of the pipette. After electroelution overnight at 2.5-5 mA, the dialysis bag was removed, sealed and subjected to dialysis against two changes of 50 mM ammonium bicarbonate at room temperature. The protein solution was lyophilized and dissolved in a minimal volume of $H_2O$.

## 2.4 Isolation of Organelle Membranes

Organelle membranes were prepared by the procedure of Fujiki *et al* (1982a). Briefly, the purified subcellular

fractions were diluted 50-fold with 100 mM $Na_2CO_3$ (pH 11.5), placed on ice for 30 minutes, then centrifuged at 225,000 x $g_{max}$ in a Beckman Type 65 rotor for 1 hour. Following centrifugation, the supernatant was decanted and the membrane pellet was rinsed with cold, distilled $H_2O$ and resuspended in $H_2O$ by sonication (Sonifier Cell Disruptor, Model W1401, Heat Systems-Ultrasonics Inc.) before protein estimations or SDS-PAGE analysis.

## 2.5 Preparation and Characterization of Antisera

### 2.5.1 Preparation of Antisera

Rat liver peroxisomal membrane pellets (approximately 300 μg protein) were resuspended in distilled water, and rat liver peroxisomal thiolase (approximately 100 μg) was isolated from purified peroxisomes by preparative SDS-PAGE (section 2.3.3). Each was emulsified in an equivolume of Freund's complete adjuvant. The suspensions were injected into the popliteal lymph nodes of both hind legs of anesthetized rabbits as described by Goudie *et al* (1966). Three subcutaneous injections (approximately 200 μg protein) were given 5 weeks later and animals were bled thereafter at 2 week intervals. The animals were boosted with three subcutaneous injections (approximately 200 μg protein) as the antibody titer decreased.

2.5.2 Immunoblot Analysis

Immunoblot analysis was performed according to the procedure of Burnette (1981) as modified by Fujiki *et al* (1984). Protein samples were separated on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose in a Bio-Rad Transblot apparatus filled with electrophoretic transfer solution (20 mM Tris base, 150 mM glycine and 20% methanol). Transfer was accomplished at 100 mA for 24-48 hours. Immmediately following transfer, the nitrocellulose was blocked in a solution of Tris-buffered saline [0.9% NaCl, 10 mM Tris-HCl (pH 7.4)] containing 1% hemoglobin for 30 minutes at 37°C with gentle agitation. The nitrocellulose was then transferred to a fresh solution of Tris-buffered saline with 1% hemoglobin containing the appropriate antiserum (1:100-1:1000 dilution) and incubated for 90 minutes at room temperature on a rocking platform (Tek-Pro Tube Rocker, Hospital Supply Corp.). The blot was washed once for 10 minutes in Tris-buffered saline and twice for 20 minutes each in Tris-buffered saline containing 0.05% (w/v) Nonidet P-40 (NP-40) and finally for 10 minutes in Tris-buffered saline. The nitrocellulose was then placed in a sealing bag containing Tris-buffered saline with 1% hemoglobin and 0.5 μCi/ml $^{125}$I-protein A for 30 minutes at room temperature with agitation. The blot was washed as above, allowed to dry, wrapped in saran wrap and exposed at -70°C to Kodak X-Omat AR-5 film (Eastman Kodak Company) in the presence of a Dupont Cronex Lightning

Plus intensifying screen (Picker International Canada Inc.).

### 2.5.3 Affinity Purification of Monospecific Antibodies From Heterogeneous Antiserum

Monospecific antibodies to the individual peroxisomal IMPs (15 kDa, 22 kDa, 36 kDa, 50 kDa, 69 kDa and 140 kDa) were affinity-purified from the heterogeneous antiserum by the antibody select procedure of Hall *et al* (1984). The IMPs were separated by SDS-PAGE and subsequently transferred to nitrocellulose paper. The nitrocellulose strip corresponding to the relative mobility of the antigen of interest was removed and treated with heterospecific antiserum (approximately 1 ml). After extensive washing of the strip in Tris-buffered saline, the monospecific antibodies were eluted from the nitrocellulose with 0.1 M glycine (pH 2.6) containing 0.15 M NaCl. This solution was quickly neutralized using 1.0 M Tris-HCl (pH 8.0), dialyzed overnight against Tris-buffered saline and concentrated by lyophilization.

### 2.5.4 Immunofluorescence Microscopy

Immunofluorescence microscopy using affinity purified IMP antibodies was carried out by Dr. Gilbert-André Keller, in the laboratory of Dr. John Singer in the Department of Biology at the University of California at San Diego. Immunofluorescence was performed essentially as described by Keller *et al* (1987). Briefly, CV-1 monkey kidney cells, grown on coverslips, were

fixed in 3% (v/v) formaldehyde in phosphate-buffered saline (PBS) (pH 7.2) for 15 minutes. The cells were permeabilized with 1% (w/v) Triton X-100 for 5 minutes followed by extensive washing with PBS. Affinity-purified antibodies with specificity to one of the 22 kDa, 36 kDa, and 50 kDa IMPs or to rabbit anti-bovine catalase were applied to the cells for 10 minutes at a concentration of 10 µg/ml. The cells were washed and treated with rhodamine-conjugated goat anti-rabbit antibodies for 10 minutes. After washing, the cells were mounted in 90% (w/v) glycerol in 100 mM Tris-HCl (pH 8.5). Immunofluorescence microscopy was performed on a Zeiss Photoscope III microscope.

## 2.6 Isolation of RNA and Cell-Free Translation

### 2.6.1 Preparation of Total RNA From Rat Liver

Total RNA was isolated from untreated or clofibrate-treated Sprague-Dawley rat liver as described by Shore and Tata (1977). The rat liver was homogenized in 200 mM Tris-acetate (pH 8.5), 50 mM potassium acetate, 10 mM magnesium acetate, 6 mM ß-mercaptoethanol, 20 µg/ml cycloheximide and 0.35 M sucrose to yield a tissue homogenate of 12.5% (w/v). The homogenate was subjected to centrifugation (17,500 x $g_{av}$) for 10 minutes at 4°C in a Sorvall SS-34 rotor. The supernatant was made to 1% SDS and 2 mM EDTA and extracted 3 times with an equivolume of phenol saturated with 10 mM EDTA (pH 7.4) and once with chloroform:isoamyl alcohol (50:1, v/v).

Subsequently, the RNA was precipitated from the aqueous phase with 0.1 volume of 2 M potassium acetate (pH 5.5) and 2.5 volumes of ethanol at -20°C. The precipitates were washed twice with 70% ethanol containing 0.1 M sodium acetate (pH 6.0), once with 70% ethanol, dried, dissolved in sterile $H_2O$ and stored at -70°C. The RNA was quantitated by spectrophotometric measurement of $OD_{260}$ and $OD_{280}$. An $OD_{260}$ of 1 corresponds to 40 µg/ml for RNA (Maniatis *et al*, 1982). The ratio of $OD_{260}$:$OD_{280}$ provided estimates of sample purity with the ideal ratio for RNA being 2.0 (Maniatis *et al*, 1982).

### 2.6.2 Preparation of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA was isolated from RNA preparations by oligo(dT) cellulose chromotography essentially as described by Maniatis *et al* (1982) with the following modifications. A one ml column of oligo(dT) cellulose was prepared by prewashing with 30 ml of binding buffer [10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA and 0.5% SDS] followed by 30 ml of elution buffer [10 mM Tris-HCl (pH 7.5) and 0.2% SDS] and finally an additional 30 ml of binding buffer. The RNA was made to 1 - 10 mg/ml in 0.5 M NaCl, 1 mM EDTA and 0.5% SDS. The RNA sample was run through the column 3 times. The RNA was heated at 65°C for 5 minutes and quick-cooled on ice-$H_2O$ prior to each passage. Subsequently, the column was washed with 30 ml of binding buffer and the poly(A)$^+$ RNA was eluted from the column using 3 ml of elution buffer. The mRNA was precipitated with

0.1 volume 2 M potassium acetate (pH 5.5) and 2.5 volumes ethanol at -20°C overnight, pelleted and washed twice with 70% ethanol. The RNA pellet was dried, dissolved in sterile $H_2O$, quantitated by $OD_{260}/OD_{280}$ and stored at -70°C.

2.6.3 Isolation of RNA From Free and Membrane-Bound Rat Liver Polysomes

Free and membrane-bound polysomes were isolated from the livers of clofibrate-treated Sprague-Dawley rats by the procedure of Ramsey and Steele (1976) as modified by Rachubinski *et al* (1980). Rat livers were homogenized (1:8 w/v) in 0.25 M sucrose containing polysome buffer [0.2 M Tris-acetate (pH 8.5 at 4°C), 0.075 M potassium acetate, 0.01 M magnesium acetate, 0.006 M ß-mercaptoethanol, 5 µg/ml cycloheximide and 150 U/ml heparin]. Membrane-bound polysomes were sedimented by centrifugation for 2 minutes at 740 x $g_{max}$ followed by 12 minutes at 131,000 x $g_{max}$ in a Beckman SW27 rotor at 4°C. Free ribosomes were pelleted from the supernatant by isopycnic centrifugation at 174,000 x $g_{max}$ for 20 hours at 4°C (Beckman Type 65 rotor) on discontinuous sucrose gradients comprised of 3 ml of each of 1.38 M and 2.0 M sucrose in polysome buffer.

RNA was isolated from the polysomes by the guanidine thiocyanate-CsCl procedure described by Raymond and Shore (1979). The free or membrane-bound polysome pellets were homogenized in 4 M guanidine thiocyanate, 0.1 M ß-

mercaptoethanol, and 25 mM sodium citrate (pH 7.0), layered over 2.75 ml of 5.7 M CsCl, 100 mM EDTA (pH 7.0) and centrifuged for 20 hours at 125,000 x $g_{max}$ in a Beckman SW41 rotor at 20°C. The pellet was dissolved in $H_2O$ and RNA precipitated by adding 0.1 volume of 2 M potassium acetate (pH 5.5) and 2.5 volumes of ethanol at -20°C. The pellet was washed twice with 70% ethanol containing 0.1 M sodium acetate (pH 6.0) and once with 70% ethanol, dissolved in $H_2O$ and stored at -70°C.

### 2.6.4 Cell-Free Translations

Cell-free translations of RNA were performed with micrococcal nuclease-treated rabbit reticulocyte lysate (Pelham and Jackson, 1976) purchased from Promega Corporation. Translations were performed according to the manufacturer's instructions for 60 minutes at 30°C using L-[$^{35}$S]methionine (1000-1500 $\mu$Ci/mmol) as the labeled amino acid. The RNA was heated at 60°C for 5 minutes and rapidly cooled in ice-water immediately prior to translation (Mortensen *et al*, 1984). The incorporation of radiolabeled methionine into newly synthesized protein was determined by the procedure of Mans and Novelli (1961). Typically, 1 $\mu$l of the translation reaction was diluted 1:20 with NP-40 mix [10 mM Tris-HCl (pH 8.5), 150 mM NaCl, 200 mM methionine, 0.02% (w/v) $NaN_3$, 1% (w/v) NP-40] and 20 $\mu$l of this diluted mixture was spotted, in duplicate, onto 1 cm x 1 cm disks of Whatman 3 mm

chromatography paper. The disks were placed into ice-cold 10% (w/v) trichloroacetic acid (TCA) containing 20 mM methionine for 20 minutes, followed by 20 minutes in boiling 5% (w/v) TCA containing 20 mM methionine. Finally, the disks were washed four times with 5% (w/v) TCA, twice with absolute ethanol and immersed in boiling ether for approximately 5 minutes. The filters were air-dried and counted in aqueous counting scintillant. The patterns of labeled polypeptides were determined by SDS-PAGE (section 2.3.2) followed by fluorography (section 2.6.5) (Bonner and Laskey, 1974).

### 2.6.5 Fluorography

Radiolabeled polypeptides in SDS-polyacrylamide gels were detected by the signal enhancement procedure of Bonner and Laskey (1974) with some modifications. Following electrophoresis, the gels were stained and destained as usual (section 2.3.2). After destaining, the gels were dehydrated by gentle agitation in dimethylsulfoxide (DMSO) for 1 hour, replacing the DMSO with fresh DMSO after 30 minutes. The gels were then placed in DMSO containing 22.2% (w/v) 2,5-diphenyloxazole (PPO) for 3 hours with gentle agitation. Subsequently, the gels were immersed in distilled $H_2O$ for 20 minutes and dried under vacuum at 60°C on a Bio-Rad gel dryer. The fluorographs were exposed to Kodak X-Omat AR-5 film at -70°C.

2.7 Immunoprecipitation of Cell-Free Translation Products

Cell-free translation products were immunoprecipitated with various antisera as described by Fujiki *et al* (1984). The translation mixtures were diluted 10-fold and adjusted to 1% (w/v) Nonidet P-40, 10 mM Tris-HCl (7.4), 150 mM NaCl, 10 mM methionine, 25 μg/ml leupeptin, 25 μg/ml antipain, 12.5 μg/ml chymostatin and 12.5 μg/ml pepstatin. The diluted translation mixtures were centrifuged at 4°C for 1 hour at 150,000 x $g_{max}$ in a Beckman Type 65 rotor. The post-ribosomal supernatants were adjusted to 0.1% SDS and 2 mM EDTA, incubated with antiserum (1:25 dilution) for 90 minutes at room temperature and then overnight at 4°C. The antibody-antigen complexes were adsorbed onto inactivated *Staphylococcus aureus* cells, prewashed with binding buffer [10 mM Tris-HCl (pH 7.4), 1% (w/v) Nonidet P-40, 0.1% SDS, 150 mM NaCl, 10 mM methionine, 2mM EDTA containing the protease inhibitors given above], for 90 minutes at room temperature. The *S. aureus* cells were pelleted by centrifugation for 1 minute in an Eppendorf micro centrifuge (model 5414, Brinkman Instruments Inc.) and washed three times with binding buffer, followed by two washes with 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% (w/v) Nonidet P-40, 10 mM methionine containing protease inhibitors, followed by one wash with 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM methionine and protease inhibitors, and finally one wash with 10 mM Tris-HCl (pH 7.4), 10 mM methionine plus protease inhibitors. The

immunocomplexes were dissociated by boiling for 5 minutes in 25 µl of SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by fluorography. The fluorographs were exposed to Kodak X-Omat AR-5 film at -70°C. The specificity of the immunoprecipitation was determined by competition of the radiolabeled polypeptide with unlabeled polypeptide which was electroeluted from SDS-polyacrylamide gel slices (section 2.3.3) and added in excess (20 µg) during the immunoprecipitation.

## 2.8 Screening the λgt11 Rat Liver cDNA Library

Thiolase antiserum and the affinity-purified anti-50 kDa IMP fraction were used to select cDNA recombinants from a λgt11 rat liver cDNA library (Clontech Laboratories Inc.). The cDNA was cloned into the unique *Eco*RI restriction site in λgt11, creating a fusion with the ß-galactosidase structural gene, *lacZ*. This facilitated screening by the double-antibody technique of Young and Davis (1983) as modified by Huynh *et al* (1985). *E. coli* Y1090 bacterial cells were grown to stationary phase in LB medium (pH 7.5). Phage particles were diluted in phage dilution buffer [10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$], and $10^5$ plaque forming units (pfus) were mixed with 0.1 ml of the Y1090 culture for 15 minutes at 37°C. The infected cells were added to 2.5 ml of molten LB soft top agar (0.7% agar) and poured onto an LB agar plate (85 mm). The plates were incubated at 42°C for a 3.5 hour growth period.

The plates were overlaid with nitrocellulose filters (BA85 nitrocellulose disks, Schleicher and Schuell) saturated with 10 mM IPTG to induce *lacZ* gene expression and then shifted to 37°C for 3.5 hours. During this time the proteins released from lytically infected cells, including the ß-galactosidase fusion proteins, were immobilized on the nitrocellulose filter. The filters were then marked, removed from the plates and blocked with a solution of 1% BSA in Tris-buffered saline + Tween 20 (TBST) [10mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% (w/v) Tween 20] for 15 minutes. The filters were first incubated with anti-peroxisomal antibodies (1:1000 dilution for anti-thiolase and 1:200 dilution for affinity-purified anti-50 kDa IMP) in TBST for 30 minutes and washed with TBST three times for 5 to 10 minutes each. Subsequently, the filters were treated with a mouse anti-rabbit IgG(Fc) alkaline phosphatase conjugate (1:7500 dilution) in TBST for 30 minutes and washed as above. Positive recombinants were detected by colourimetric development using the substrates nitro blue tetrazolium (NBT, 0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.165 mg/ml) in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$. Immunoreactive phage plaques were selected and purified to homogeneity by subsequent replatings and rescreenings.

## 2.9 Preparation of Fusion Protein Extracts From λgt11 Lysogens

To obtain large amounts of the recombinant ß-galactosidase fusion proteins from λgt11 recombinants, bacterial cell lysates were made. This was done by infecting the *E. coli* strain Y1089, a high frequency lysogenic host. During lysogenic growth transcription of the viral late genes (responsible for coat protein production and packaging) is repressed, and production of the ß-galactosidase fusion protein can be induced by IPTG without subsequent formation of progeny bacteriophage particles and cell lysis (Huynh *et al*, 1985).

*E. coli* Y1089 cells were grown to saturation in LB medium containing 0.2% maltose at 37°C. The Y1089 cells were infected at a multiplicity of approximately 5 for 20 minutes at 32°C in LB medium containing 10 mM $MgCl_2$. The cells were plated on LB plates at a density of approximately 200 per plate and incubated at 32°C. At this temperature, the temperature-sensitive phage repressor is functional, blocking expression of phage gene products which are necessary for lytic growth and thereby favouring lysogenic growth. These colonies were picked onto two LB plates, one of which was incubated at 42°C, the other at 32°C. The colonies which grew at 32°C but not at 42°C were assumed to be lysogens.

In order to prepare a crude lysate from the λgt11 lysogens, 100 ml of LB medium was inoculated with a single

colony of the Y1089 recombinant lysogen and incubated at 32°C. When the culture acheived an $OD_{600}$ of 0.5, the incubation temperature was rapidly raised to 42°C-45°C for 20 minutes. Subsequently, IPTG was added to a final concentration of 10 mM and the culture was incubated for 45-60 minutes at 37°C. The cells were harvested in a Sorvall SS-34 rotor at 5,000 rpm for 5 minutes at room temperature. The cells were resuspended in 2 ml of TEP buffer [100 mM Tris-HCl (pH 7.4), 10 mM EDTA, 1mM phenylmethylsulfonylfluoride (PMSF)] and frozen in liquid nitrogen. Thawing the frozen cells resulted in lysis, and sonication (Sonifier Cell Disruptor, Model W1401, Heat Systems-Ultrasonics Inc.) was used to ensure complete lysis. The lysis extract was centrifuged at 10,000 rpm for 10 minutes, and both the supernatant fraction and the pellet fraction (containing bacterial membranes and unbroken cells) were kept for analysis (Huynh *et al*, 1985).

## 2.10 Preparation of Phage DNA and Subcloning into Plasmids

### 2.10.1 Preparation of Phage DNA

DNA was isolated from phage by the plate lysis method (Maniatis *et al*, 1982). A single plaque was placed into 1 ml of SM buffer [0.1 M NaCl, 8mM $MgSO_4.7H_2O$, 50 mM Tris-HCl (pH 7.5) and 0.01% gelatin] containing a drop of chloroform and incubated for 4 to 6 hours at 4°C. Y1090 bacterial cells were grown to saturation and 0.1 ml of bacterial suspension was incubated with 0.1 ml of the phage suspension (approximately

$10^5$ pfu) for 20 minutes at 37°C. The infected cells were added to 2.5 ml of molten LB soft top agarose (0.7% agarose) and poured onto the surface of an LB agar plate (85 mm). The plate was incubated at 37°C for 9-14 hours. SM buffer was poured onto the plate (5 ml per plate), and the phage were eluted at room temperature for 1-2 hours with gentle agitation. The liquid was removed from the plate and centrifuged in a Sorvall SS-34 rotor at 8000 x $g_{max}$ for 10 minutes at 4°C to remove bacterial debris. The supernatant was treated with 1 µg/ml of each of RNase A and DNase I for 30 minutes at 37°C. The bacteriophage particles were precipitated with an equivolume of 20% (w/v) polyethylene glycol and 2 M NaCl in SM for 1 hour at 0°C and recovered by centrifugation in a Sorvall SS-34 rotor at 10,000 x $g_{max}$ for 20 minutes at 4°C. The pellet was resuspended in 0.5 ml SM, made to 0.1% SDS and 5 mM EDTA (pH 8.0) and incubated for 15 minutes at 68°C. The phage DNA was extracted once with phenol, once with phenol:chloroform: isoamyl alcohol (50:50:1, v/v/v) and once with chloroform: isoamyl alcohol (50:1, v/v), precipitated with an equivolume of isopropanol and washed with 70% ethanol.

2.10.2 Subcloning of cDNA Inserts From Phage into Plasmids

Isolated phage DNA was digested with the restriction endonuclease *Eco*RI to liberate the cDNA inserts. These cDNA fragments were isolated from agarose gels by electroelution

(section 2.12.4) and inserted into the *Eco*RI site of pUC based plasmids [pUC118 or pGEM-7Zf(+)]. Generally, ligations were performed as outlined in the cloning strategies section of the International Biotechnologies catalogue. Typically, ligations were conducted overnight at 16°C using molar ratios of insert:vector of 2:1. Ligations were performed in either T4 DNA ligase buffer [50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% (w/v) polyethylene glycol (PEG)-8000] (Gibco/BRL) or in 0.5X potassium glutamate buffer (KGB) [50 mM potassium glutamate, 12.5 mM Tris-acetate (pH 7.6), 5 mM magnesium acetate, 25 μg/ml BSA, 0.25 mM β-mercaptoethanol] supplemented with 1 mM ATP (Hanish and McClelland, 1988).

## 2.11 Transformations of *E. coli* With Plasmid DNA

Competent *E. coli* DH5α cells were purchased from Gibco/BRL, and transformations were carried out as described in the product data sheets. Briefly, the DH5α cells were thawed on ice and 50 μl aliquots were placed into chilled microcentrifuge tubes. Transformations were carried out with 10 ng of control plasmid or 1-3 μl of the DNA ligation reaction mixtures. The DNA was mixed with the cells and incubated on ice for 30 minutes. The mixtures were heat-shocked at 37°C for 20 seconds and placed onto ice for 2 minutes. After addition of 0.95 ml of LB medium, the tubes were incubated at 37°C for one hour with agitation. Appropriate dilutions of the transformation mixture (typically

0.1 and 0.9 volumes) were spread onto LB agar plates containing ampicillin (100 μg/ml) which were previously treated with 60 μl of 2% X-gal in dimethylformamide. The agar plates were incubated overnight at 37°C.

## 2.12 Preparation of Plasmid DNA

Plasmid DNA was extracted from bacterial cell cultures by the alkaline lysis method of Birnboim and Doly (1979) as outlined in Maniatis *et al* (1982). When large quantities of highly purified plasmid DNA were required, the large-scale isolation procedure was followed (Maniatis *et al*, 1982). Briefly, the alkaline lysis method of preparing plasmid DNA involved harvesting an overnight bacterial culture (1.5 ml for mini-preparations and 50-500 ml for large-scale prepations). The bacterial pellet was resuspended in 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl (pH 8.0), followed by the addition of 0.2 N NaOH and 1% SDS to lyse completely the cells. The lysate was neutralized by the addition of potassium acetate (3 M with respect to potassium and 5 M with respect to acetate, pH 4.8). For mini-preparations, this mixture was centrifuged for 5 minutes in an Eppendorf micro centrifuge to remove the insoluble network of renatured chromosomal DNA, as well as protein-SDS complexes and high molecular weight RNA. The supernatant was extracted with phenol:chloroform:isoamyl alcohol (50:50:1, v/v/v), and the plasmid DNA was precipitated from the aqueous phase with 2 volumes of ethanol and washed

with 70% ethanol. The plasmid pellet was dried in a vacuum dessicator and dissolved in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], containing DNase-free pancreatic RNase (20 μg/ml). For large-scale plasmid preparations the centrifugation was carried out in a Sorvall SS-34 rotor at 12,000 x $g_{max}$ for 30 minutes. Plasmid DNA was precipitated from the supernatant with 0.6 volume of 2-propanol and recovered by centrifugation as before. The pellet was washed with 70% ethanol and dried in a vacuum dessicator. The DNA pellet was dissolved in TE and subjected to equilibrium density centrifugation in a cesium chloride gradient containing saturating quantities of the intercalating dye, ethidium bromide as described by Radloff *et al* (1967) and Maniatis *et al* (1982). Briefly, 1 g of solid cesium chloride was added per milliliter of DNA solution and ethidium bromide was added to a final concentration of 0.6 mg/ml. The cesium chloride solution was centrifuged at 130,000 x $g_{av}$ for 48 hours at 20°C in a Beckman Type 65 rotor or for at least 3 hours at 338,000 x $g_{av}$ in a Beckman TLV-100 rotor. Following centrifugation, the closed circular plasmid DNA was removed from the gradient with a hypodermic needle and the ethidium bromide was removed by extraction with several equal volumes of 1-butanol saturated with water. The clear aqueous phase was dialyzed against several changes of TE (pH 8.0).

## 2.13 Analysis and Purification of DNA by Electrophoresis

### 2.13.1 Agarose Gel Electrophoresis

The separation of DNA fragments by electrophoresis through agarose gels was performed as outlined by Maniatis *et al* (1982). Typically, 1% agarose gels were run in 1X TBE buffer [0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA (pH 8.0)]. Ethidium bromide (0.5 μg/ml) was added both to the gel and to the running buffer to allow visualization of the DNA fragments in the gel by UV-illumination. The gels were cast as minigels (6.5 cm x 10 cm) or larger gels (14.5 cm x 20 cm or 14.5 cm x 25 cm) in Hoefer horizontal agarose submarine gel casting trays (Hoefer Scientific Instruments). Samples in gel-loading dye [0.04% bromophenol blue, 0.04% xylene cyanol and 6.67% (w/v) sucrose] were loaded into the wells of the submerged gel, and the gel run in the Hoefer horizontal agarose submarine electrophoresis apparatus at 5-10 V/cm.

The DNA in the gel was visualized by midrange ultraviolet light (wavelength = 300 nm) using a Fotodyne UV 300 "plus" transilluminator (Bio/Can Scientific Inc.) and photographed using the Fotodyne FCR-10 DNA photographic system (Bio/Can Scientific). Alternatively, DNA was visualized using the Fotodyne fotosystem 1000 UV transilluminator (wavelength = 300 nm) and photographed using a Polaroid MP-4 land camera. Photographs were taken with Polaroid 667 Coaterless Instant Film (Bio/Can Scientific Inc.).

### 2.13.2 Non-Denaturing Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis used to analyze and prepare fragments of DNA was performed as described by Maniatis *et al* (1982). The gels were cast in a variety of polyacrylamide concentrations (3.5%-10%) using acrylamide:N,N'-methylene-bis-acrylamide (29:1, w/w). The Hoefer vertical slab gel unit (model SE 400, Hoefer Scientific Instruments) was used to cast and run gels. Samples were prepared in gel-loading dye [0.04% bromophenol blue, 0.04% xylene cyanol and 6.67% (w/v) sucrose], and electrophoresis was carried out at 1-8 V/cm. Following electrophoresis, the gel was submerged in staining solution (1X TBE buffer containing 0.5 μg/ml of ethidium bromide) for 30 minutes and the DNA visualized on a UV-transilluminator as described previously (section 2.12.1).

### 2.13.3 Denaturing Polyacrylamide Gel Electrophoresis

To analyze separate strands of denatured duplex DNA, the DNA was subjected to electrophoresis through denaturing polyacrylamide gels as described by Maniatis *et al* (1982). The only modification to the protocol was that the DNA sample was prepared in 38% formamide, 8 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol, heated for 5 minutes at 90°C and quick-cooled on ice prior to loading onto the gel. The gels were cast as 3.5% to 5% polyacrylamide containing 8 M urea (Maniatis *et al*, 1982). Electrophoresis was carried out at

10-20 V/cm in a Hoefer vertical slab gel electrophoresis apparatus until the marker dyes migrated a suitable distance.

### 2.13.4 Purification of DNA from Gel Fragments by Electroelution

DNA was purified from agarose or acrylamide gel slices by electroelution using the unidirectional electroelutor (model UEA; International Biotechnologies Inc.) as described in the operator's manual. Briefly, DNA fragments of interest were excised from either agarose (GTG agarose, FMC BioProducts) or acrylamide gels using a razor blade, and gel slices were placed into the slots of the electroelutor containing 0.5X TBE buffer. DNA was eluted from the gel slices for approximately 1 hour at 80-100 volts and trapped in high salt buffer (7.5 M ammonium acetate containing trace bromophenol blue) in the V-channel of the electroelutor. The DNA was recovered and precipitated with ethanol as described in the unidirectional electroelutor operator's manual.

## 2.14 Labeling DNA Probes

### 2.14.1 5'-end Labeling of Oligodeoxyribonucleotides

Oligodeoxyribonucleotides (6 pmol) were 5'-end-labeled by incubation with 10 $\mu$Ci [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol) and 3-5 units of T4 polynucleotide kinase in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and 5 mM DTT for 40 minutes at 37°C in a 30 $\mu$l reaction volume. Incorporation of $^{32}$P was quantitated by

spotting 1 µl of labeling reaction on DE81 paper and determining counts remaining after three washes in 0.01 M $Na_2HPO_4$, 0.001 M $Na_2H_2P_2O_7$, of 5 minutes each at room temperature. Typically, the specific activity of the oligodeoxyribonucleotides was 2.5-5 x $10^7$ cpm/µg.

2.14.2 Labeling cDNA Probes

Following restriction endonuclease digests, the DNA of interest was electrophoresed in 1% low melting point (LMP) agarose containing 40 mM Tris-acetate (pH 8.0), 1 mM EDTA and 5 mM sodium acetate. The DNA fragment to be labeled was excised and diluted with 1.5 volume of $H_2O$ to yield a final DNA concentration of approximately 1 ng/µl. The sample was boiled for 7 minutes and divided into 25 µl aliquots. Aliquots to be labeled immediately were kept at 37°C. The remaining aliquots were stored frozen at -20°C and reboiled for 3 minutes just prior to labeling. The DNA fragments were labeled as described by Feinberg and Vogelstein (1983; 1984) using the random primer DNA labeling kit (BRL). The reaction mixture included approximately 20 ng of DNA in 0.67% LMP agarose, 20 µM of each dCTP, dGTP and dTTP, 50 µCi of [α-$^{32}$P]dATP (3,000 Ci/mmol, 10 µCi/µl), 5.4 $OD_{260}$ units/ml oligodeoxyribonucleotide hexamer primers ($(dN)_6$), in random primer buffer (200 mM HEPES, 50 mM Tris-HCl, 5mM $MgCl_2$, 2mM ß-mercaptoethanol, 0.4 mg/ml BSA, pH 6.8). The labeling reaction was initiated by the addition of 2-5 units of Klenow

fragment and proceeded for 1-20 hours. The labeled probe was separated from unincorporated nucleotides by spun column chromotography using sephadex G-50 (Maniatis *et al*, 1982). Typically, the specific activity of a DNA fragment was 5-10 x $10^6$ cpm/$\mu$g.

2.14.3 Labeling the 1 kbp Ladder

The 1 kbp ladder (BRL) was used as molecular weight markers for all DNA and RNA analyses. When necessary the DNA fragments were $^{32}$P-end-labeled by the phosphate exchange reation. The reaction mixture included: 2 $\mu$g of 1 kbp ladder, 20 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol), 50 mM imidazole (pH 6.4), 12 mM $MgCl_2$, 1 mM DTT, 0.3 mM ADP, 0.5 $\mu$M ATP and 20 units of T4 polynucleotide kinase. This mixture was incubated at 37°C for 30 minutes, and the reaction was stopped by the addition of EDTA to 20 mM, followed by extraction with phenol: chloroform:isoamyl alcohol (50:50:1, v/v/v). The DNA fragments were precipitated in 0.3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol, washed in 70% ethanol, dried and dissolved in TE 7.5 (25$\mu$l). Typically, the specific activity of the labeled ladder was 5-10 x$10^5$ cpm/$\mu$g DNA, and 10,000 cpm loaded in one lane of a gel was sufficient to be detected overnight with Kodak X-Omat AR-5 film.

## 2.15 Analyses of DNA by Blotting and Hybridization

### 2.15.1 Southern Blot Analysis

Southern blots (Southern, 1975) were performed essentially as outlined by Ausubel *et al* (1989). Following electrophoresis of DNA fragments in an agarose gel, the gel was placed in a tray of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes with gentle agitation and subsequently neutralized by treatment with 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.6) for 30 minutes with gentle agitation. The transfer was carried out either by the capillary transfer technique for 12-20 hours described by Ausubel *et al* (1989) or by the positive pressure blotting technique using the PosiBlot Pressure Blotter (Stratagene). Routinely, transfer was carried out using 10X SSC [1.5 M NaCl, 0.15 M trisodium citrate (pH 7.0)]. After the transfer was complete, the nitrocellulose was air-dried and baked *in vacuo* for 2 hours at 80°C using a Napco vacuum oven (model 5831; Fisher Scientific) or the DNA was covalently bound to the nitrocellulose by ultraviolet irradiation (0.12 J/cm$^2$) using a UV Stratalinker 1800 (Stratagene). For blots probed with oligodeoxy-ribonucleotides, prehybridizations and hybridizations were performed in 5X SSC, 5X Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.01 M $Na_2HPO_4$ and 0.001 M $Na_2H_2P_2O_7$ (Harley, 1987). Prehybridizations were for three hours at room temperature, and hybridizations were overnight at room temperature with 4 x 10$^5$ cpm/ml of $^{32}$P-

end-labeled oligodeoxyribonucleotides. Specific activity of the oligodeoxyribonucleotides was approximately 5 x $10^5$ cpm/pmol primer. Blots were washed three times for 15 minutes each in 2X SSC at 35°C. For blots probed with cDNA probes, prehybrizations were performed in 1.25X SSC, 0.16X Denhardt's solution, 4 μg/ml denatured salmon sperm DNA, 0.001% (w/v) SDS and 0.02 M sodium phosphate (pH 7.0) at 65°C for 3 hours. Hybridizations were in the same solution containing 30% (v/v) de-ionized formamide and 4 x $10^5$ cpm/ml of radioactive probe at 50°C for 12-20 hours. Washing was performed three times for 15 minutes each at 55°C in 1X SSC (Murray and Rachubinski, 1987). All radiolabeled probes were boiled for 10 minutes and quick-cooled on ice prior to hybridization.

2.15.2 Bacterial Colony Hybridization

*E. coli* colonies carrying different recombinant plasmids were screened to determine which plasmids contained a specific DNA sequence by the colony hybridization procedure (Grunstein and Hogness, 1975; Hanahan and Meselson, 1980). Bacterial cells were grown on LB agar plates containing the appropriate antibiotic until colonies were about 0.1-1 mm in diameter (typically 10 hours at 37°C). A nitrocellulose filter was laid onto the plate and its orientation marked with a needle. The filter was peeled off the plate and placed with bacterial colonies up in 3 ml of 0.5 M NaOH layered on saran wrap. After 3 minutes, the filter was blotted briefly onto

paper towels, and the process was repeated once again with 0.5 M NaOH, twice with 1 M Tris-HCl (pH 7.6) and twice with 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4). Subsequently, the filter was placed in 5 ml of 2X SSC on saran wrap for 5 minutes and colony debris was wiped away using clean gloves. The nitrocellulose filters were baked *in vacuo* or UV irradiated, prehybridized, hybridized and washed as previously described (section 2.15.1).

## 2.16 Nucleotide Sequence Determination

Double-stranded dideoxy-chain termination sequencing was performed as described Sanger *et al* (1977) and Zhang *et al* (1988). Plasmid DNA was purified on CsCl gradients, and 3 μg of template DNA was denatured in 0.2 N NaOH containing 0.2 mM EDTA at room temperature for 5 minutes. This mixture was neutralized by adding ammonium acetate (pH 4.6) to 0.2 M, and the DNA was precipitated with 3 volumes of ethanol (-20°C, 15 minutes). The DNA was pelleted in an Eppendorf micro centrifuge, washed with 70% ethanol and dried in a vacuum desicator (Speed Vac Concentrator, Savant Instruments Inc.). The DNA was dissolved in distilled $H_2O$, and dideoxy-chain termination sequencing was carried out with chemically modified T7 DNA polymerase (Tabor and Richardson, 1987) using the Sequenase™ DNA sequencing kit (United States Biochemical Corporation). The labeled nucleotide in the sequencing reaction was [$\alpha$-$^{32}$P]dATP (3,000 Ci/mmol), and the only

modification to the Sequenase™ protocol was that 1 pmol of sequencing primer was used in each annealing reaction. The sequencing reactions were resolved on 6% acrylamide sequencing gels containing 8 M urea (Gibco/BRL M13 Cloning/Dideoxy-sequencing Instruction manual). After electrophoresis, gels were dried and exposed to Kodak X-Omat K film overnight.

## 2.17 Construction of Exonuclease III Nested Deletion Fragments

Large segments of DNA were sequenced by the targeted breakpoint DNA sequencing strategy of Henikoff (1984). The only modification to the protocol was that the starting material was 10 μg of CsCl-purified plasmid DNA. Nested deletions were constructed by unidirectional digestion with exonuclease III, exactly as described by Henikoff (1984), allowing sequential DNA sequence analysis by the double-stranded dideoxy-chain termination sequencing procedure (Zhang *et al*, 1988) (section 2.16).

## 2.18 Analyses of RNA

### 2.18.1 Northern Blot Analysis

Northern blot analysis was performed essentially as described by Ausubel *et al* (1989). Poly(A)$^+$ RNA, isolated from the livers of normal and clofibrate-treated rats, was denatured at 55°C for 10-15 minutes in sample buffer containing 20 mM sodium borate (pH 8.3), 0.2 mM EDTA, 6.5%

formaldehyde, 42.5% formamide, 0.04% bromophenol blue, 0.04% xylene cyanol and 6.7% sucrose. The RNA (5 μg per lane) was separated by electrophoresis in formaldehyde-agarose gels [1% agarose, 20 mM sodium borate (pH 8.3), 0.2 mM EDTA and 1.1% formaldehyde] at a constant voltage of 5 V/cm for about 3 hours or until the bromophenol blue band migrated halfway down the gel. The gels were washed several times with $H_2O$ to remove formaldehyde and placed in a tray of 10X SSC for 45 minutes with gentle agitation. RNA was transferred from the agarose gels to nitrocellulose using the capillary transfer method in 20X SSC (3M NaCl, 0.3 M trisodium citrate) for 12-20 hours. After transfer the blots were air-dried, baked in a vacuum oven for 2 hours at 80°C or subjected to UV irradiation, prehybridized, hybridized and washed as previously described (section 2.15.1).

### 2.18.2 RNA Slot Blot Analysis

Poly(A)$^+$ RNA was isolated from untreated and clofibrate-treated rat liver as outlined in section 2.6.2. A serial dilution of poly(A)$^+$ RNA (0.1 ng/μl to 10 ng/μl) was prepared in a solution of 3X SSC, 5 mM methyl mercury hydroxide and 50 μg/ml calf liver tRNA. The slot blot apparatus (Minifold II Slot-Blot System, model SRC072/0, Schleicher and Schuell Inc.) was assembled as described in the owner's manual, and the nitrocellulose and 3 mm paper were soaked in 3X SSC prior to assembly. One-hundred microliter

aliquots of each dilution of RNA were applied to the wells of the slot blot filtration apparatus while connected to a vacuum source. Following sample application, the nitrocellulose was air-dried and baked *in vacuo* at 80°C for 2 hours or subjected to ultraviolet irradiation (0.12 J). Prehybridization, hybridization and wash conditions were as previously described (section 2.15.1). To confirm that equal amounts of poly(A)$^+$ RNA were bound to nitrocellulose in adjacent lanes of the slot blot, the blots were quantitated by binding of radiolabeled oligo(dT)$_{18}$ (Harley, 1987). The original radiolabeled probe was boiled off the blot in 1X SSC, 0.1% SDS (3 times for 5-10 minutes each). Oligo(dT)$_{18}$ was $^{32}$P-end-labeled in a reaction containing 240 pmol oligo(dT)$_{18}$, 10 $\mu$Ci [$\gamma^{32}$P]ATP (3,000 Ci/mmol), 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5mM DTT and 3-5 units of T4 polynucleotide kinase. The blots were subsequently rehybridized with 20 pmol of labeled oligo(dT)$_{18}$ per ug of bound poly(A)$^+$ RNA, washed in 2X SSC at 35°C and reexposed to Kodak X-Omat AR-5 film. Results were analyzed using a scanning densitometer (model GS300) and densitometry analysis software (GS-350) (Hoefer Scientific Instruments).

## 2.19 Primer Extension of Partial cDNA Recombinants

The 5'-ends of the cDNAs encoding thiolase 2 and the 50 kDA IMP were determined by the primer extension strategy termed "rapid amplification of cDNA ends" (RACE) as described by Frohman *et al* (1988). This procedure is outlined in

Results and Discussion, section 4.2, Fig. 4.2.4. The experimental details for the thiolase 2 cDNA are given below, and those specific to the 50 kDa IMP cDNA are outlined in the Appendix, section A.3.

Two synthetic oligodeoxyribonucleotides were constructed complementary to the coding strand and near the 5′-end of the partial cDNA recombinant for thiolase 2 (see fig. 4.2.6). One primer (AB704) was used to direct first-strand cDNA synthesis from rat liver poly(A)$^+$ RNA, and the other primer (AB616, located further upstream) was used in the polymerase chain reaction (PCR) to generate multiple copies of the specific cDNA. One microgram of poly(A)$^+$ RNA was dissolved in water, heated at 65°C for 3 minutes and quick-cooled on ice. First-strand cDNA synthesis was carried out at 42°C for 2 hours in 50 mM Tris-HCl (pH 8.3 at 42°C), 100 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, each dNTP at 1 mM, 10 units of RNasin, 20 pmol of the reverse transcriptase primer (RT primer, AB 704), 2 pmol of $^{32}$P-end labeled RT primer and 10 units of avian myeloblastosis virus reverse transcriptase. The excess RT primer was removed by gel filtration chromotography using a 2 ml Bio-Gel A-5m column (Bio-Rad) equilibrated with 0.05X TE. Forty-five one-drop fractions were collected, and the first 8 tubes containing radioactivity were pooled and concentrated by centrifugation under reduced pressure (Speed Vac Concentrator, Savant Instruments Inc.). The 5′-end of the newly-synthesized cDNA strand was tailed

using 0.2 mM dATP, 100 mM potassium cacodylate (pH 7.2), 2mM $CoCl_2$, 0.2 mM DTT and 15 units of terminal deoxynucleotidyl transferase. The mixture was incubated for 10 minutes at 37°C, and the reaction was stopped by heating at 65°C for 15 minutes. The reaction mixture was diluted to 100 µl in TE (pH 8.0), and 1- to 10-µl aliquots were used for amplification. The amplification mixture contained 1- to 10-µl aliquots of the newly synthesized cDNA strand mix, 10 pmol $(dT)_{17}$-adaptor (AB703), 25 pmol adaptor primer (AB705), 25 pmol amplification primer (AB616), 67 mM Tris-HCl (pH 8.8 at 25°C), 6.7 mM $MgCl_2$, 10 mM DTT, 16.6 mM $(NH_4)_2SO_4$, 6.7 µM EDTA, 170 µg/ml BSA, 10% DMSO and each dNTP at 1.5 mM. This mixture was denatured (5 minutes, 95°C) and cooled to 70°C. Subsequently, 2.5 units of *Thermus aquaticus* (Taq) DNA polymerase was added, and the reaction was overlaid with 50 µl of mineral oil. Annealing took place at 55°C for 2 minutes, and the second strand of cDNA was extended at 70°C for 40 minutes using a DNA Thermal Cycler (Perkin-Elmer-Cetus). Subsequently, the reaction underwent reiterated steps of denaturing (92°C, 1 minute), annealing (50°C, 2 minutes) and extension (70°C, 3 minutes) for 30 cycles. Following PCR amplification, the reactions were extracted once with chloroform:isoamyl alcohol (50:1, v/v), treated with 20 µg/ml RNase A and analyzed by agarose gel electrophoresis. The RACE reaction products were equilibrated in TE by spun column chromotography using sephadex G-50 (Maniatis *et al*, 1982). The cDNA was digested with the

restriction enzyme *Sal*I, which recognizes a site in the adaptor placed at the 5'-end of the newly synthesized cDNA (AB705), and with *Sst*II, which cuts within the known sequence of thiolase 2 cDNA, and subcloned into the vector pGEM-5Zf(+). Colony lift hybridization (section 2.15.2) using a $^{32}$P-end-labeled synthetic oligodeoxyribonucleotide probe (AB755) was used to identify plasmid harbouring the cDNA insert of interest.

## 2.20 *In vivo* Targeting of Rat Liver Thiolase to Peroxisomes in Mammalian Cells

The following experiments were performed by Dr. B.W. Swinkels and Dr. S.J. Gould in the laboratory of Dr. S. Subramani. We supplied two plasmids, one containing the cDNA encoding mature thiolase pT4-4 and one encoding the thiolase prepiece pT5'-6, as well as the thiolase antibodies used in these experiments.

### 2.20.1 Plasmid Constructions

The plasmid pRSV-An was used for the expression of the thiolase gene constructs. This vector was constructed from pRSV-CAT (Gorman *et al*, 1982a) in which the CAT (chloramphenicol acetyltransferase)-containing *Hind*III-*Bam*HI fragment had been replaced with the *Bcl*I-*Bam*HI fragment of SV40 (positions 2770-2533) containing the early region poly-adenylation signal (An). The resultant plasmid (pRSV-An)

contains several restriction endonuclease sites (*Hind*III, *Xba*I and *Bgl*II) suitable for subcloning between the RSV long terminal repeat promoter and the SV40 An signal. The plasmid pT4-4 (Bodnar and Rachubinski, 1990), containing the partial cDNA for thiolase 2, lacking about 180 bp of the full-length sequence at its 5'-end, was used as a template in PCR with the primers SGThiolase 5'-GGA AGC TTA CCA TGG CTA CCT TCC CGC AGG-3' and thiolase2 5'-GAG ATC TAG TTC CCA GGG TAT TCA AAG ACA GC-3'. The fragment obtained by PCR (approximately 1100 bp) was digested with *Hind*III and *Bgl*II and inserted into pRSV-An to generate plasmid pRSV-thiol, encoding a protein corresponding to the mature thiolase. The proteins encoded by the various fusion constructs are schematically represented in Results and Discussion, section 4.7, Fig. 4.7.1. The internal *Sfi*I-*Stu*I fragment of pRSV-thiol was replaced with that isolated from pT4-4 to exclude possible mutations resulting from PCR amplification from most of the thiolase cDNA. This recombinant, called pRSV-thiol-40 was used for further experiments.

The cDNA recombinant pT5'6 (Bodnar and Rachubinski, 1990), which contained the 5'-end of thiolase 2 cDNA, was used as a template in PCR with primers FLT-1 5'-GGG AAG CTT GCG GCC GCC ATT TTG GTT TGT TAA GC-3' and INT-1 5'-CCC TCT AGA GGG CCC GCG CGG CCG ATG GGG GTG CG-3'. The fragment derived from this PCR (approximately 240 bp) was digested with *Hind*III and *Xba*I and subcloned into the plasmid pTZ-19U (Pharmacia) to yield

plasmid pTZ-FLT1. The *Hind*III-*Sfi*I fragment containing the thiolase 2 cDNA 5′-end was subcloned into pRSV-thiol-40 to give plasmid pRSV-FLT1-thiol, which encodes the precursor form of thiolase 2 (Fig. 4.7.1).

The thiolase 2 cDNA contains a *Hinf*I site downstream of its initiation codon but upstream of its second methionine codon, which corresponds to the initiation codon of the thiolase 1 gene. The *Hinf*I site was used to remove the first initiation codon from pTZ-FLT1 to obtain pTZ-FLT2, which contains a 5′-end corresponding to that of the thiolase 1 gene. A *Hind*III-*Sfi*I fragment was isolated from pTZ-FLT2 and inserted into pRSV-thiol-40 to obtain the plasmid pRSV-FLT2-thiol, encoding a protein corresponding to the precursor of thiolase 1 (Fig. 4.7.1).

The CAT fusion constructs were created using the plasmid pSV2-Not-leader-CAT, which consists of pSV2-CAT (Gorman *et al*, 1982b) with a *Not*I site and other sites from the pUC8 polylinker immediately upstream of the CAT translational initiation site. To create the in-frame fusions of the amino terminal parts of thiolases 2 and 1 to the CAT protein, pTZ-FLT1 and pTZ-FLT2 were both digested with *Not*I and *Xba*I to release fragments containing the 5′-ends of the thiolase 2 (approximately 230 bp) and thiolase 1 (approximately 190 bp) cDNAs, respectively. These fragments were subcloned into pSV2-Not-leader-CAT to obtain pSV-FLT1-CAT and pSV-FLT2-CAT, respectively. These constructs encode the

amino terminal prepieces of thiolase 2 and 1, as well as the first 21 amino acids of mature thiolase fused to CAT (Fig. 4.7.1).

The constructs which contain deletions in the thiolase 1 prepiece fused to CAT (pRSV-FLT3-CAT, pRSV-FLT4-CAT and pRSV-FLT5-CAT) were created by PCR amplification of the CAT DNA fragment followed by subcloning. The plasmid pSV2-CAT was used as a template in a PCR with primers TCF-1 5'-CTG GCC GGC CGC TCG AGT GGT ACC ATG GAG AAA AAA ATC ACT GGA-3' and CAT-3 5'-ATG GAT CCA GAT CTT ACG CCC CGC CCT GCC-3'. The fragment obtained from this reaction (approximately 700 bp) was digested with *Eag*I and *Bgl*II and subcloned into pRSV-FLT2-thiol (previously digested with *Eag*I and *Bgl*II) to obtain pRSV-FLT3-CAT, encoding the first 15 amino acids of the thiolase 1 prepiece fused to CAT. The 700 bp fragment obtained in the PCR with primers TCF-1 and CAT-3 was diluted 10,000-fold and used as a template in a PCR with primers FLT-4 5'-GGC TGC AGG TAG TGC TGG GCC ACC TCT CGA GTG GTA CCA TGG-3' and CAT-3. The fragment obtained by this PCR (approximately 700 bp) was digested with *Pst*I and *Bam*HI and cloned into pTZ-FLT2 (digested with *Pst*I and *Bam*HI) to obtain pTZ-FLT4-CAT. This plasmid was digested with *Hind*III and *Bgl*II to release the FLT4-CAT fragment which was subsequently cloned into pRSV-An to yield pRSV-FLT4-CAT. To construct pRSV-FLT5-CAT, the diluted TCF-1/CAT-3 PCR fragment was used as a template in PCR using primers FLT-5 5'-GGA AGC TTG CCA TGC ATC GCC TGC AGT CGA

GTG GTA CCA TGG-3′ and CAT-3. The fragment obtained by this PCR (approximately 700 bp) was digested with *Hind*III and *Bgl*II and cloned into pRSV-An to obtain pRSV-FLT5-CAT (Fig. 4.7.1).

2.20.2 Transfections of Mammalian CV-1 and CV-H Px110 Cells

CV-1 and CV-H Px110 monkey kidney cells were grown on glass coverslips in 10-cm dishes as described by Keller *et al* (1987). After twenty-four hours of growth, the semiconfluent monolayers were transfected by the calcium phosphate procedure of Parker and Stark (1979). Briefly, the plasmid DNA was diluted in a solution containing 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM dextrose and 21 mM HEPES (pH 7.1). Salmon sperm DNA was then added to a final total DNA concentration of 50 μg/ml. To this solution, 2M $CaCl_2$ was added to a final concentration of 125 mM. The DNA co-precipitated with calcium phosphate during 20 to 30 minutes at room temperature. The medium was removed from the cells, and 0.5 ml of DNA suspension was added to the cells for 20 minutes at room temperature. Subsequently, fresh medium containing 5% fetal bovine serum was added, and the monlayers were incubated at 37°C for 4 hours. The supernatant fluids were removed, the monolayers were washed once with fresh medium and incubation was continued at 37°C.

2.20.3 Immunofluorescence Microscopy

Forty-eight hours after transfection

immunofluorescence microscopy was performed exactly as described in Materials and Methods, section 2.5.4. CV-H Px110 cells transfected with the thiolase constructs were incubated with rabbit anti-thiolase antibodies and the CAT2 mouse monoclonal anti-CAT antibodies, followed by treatment with a fluorescein conjugate of a goat anti-rabbit IgG antibody and a rhodamine conjugate of a goat anti-mouse IgG antibody. CV-1 cells transfected with pSV2CAT or the CAT-fusion constructs were incubated with the CAT2 mouse monoclonal anti-CAT antibody and with the rabbit anti-SKL antibodies, followed by a fluorescein conjugate of a goat anti-mouse IgG antibody and a rhodamine conjugate of a goat anti-rabbit IgG antibody.

## RESULTS AND DISCUSSION

### 3. Characterization of The Integral Membrane Polypeptides of Rat Liver Peroxisomes

Knowledge of the properties of the peroxisomal membrane is essential to understanding the biogenesis and function of this organelle. The membrane is responsible for maintaining the integrity of the organelle, controlling the flux of polypeptides, substrates, cofactors and products, as well as playing a role in formation, growth and division. Until recently only limited information was available on the composition and biophysical properties of the peroxisomal membrane. Fujiki *et al* (1982a; 1982b) developed a simple and rapid procedure for isolating membranes from purified peroxisomes which has facilitated characterization of the components of the membranes. This section describes the characterization of the integral membrane polypeptides (IMPs) of rat liver peroxisomal membranes isolated by this procedure. The coordinated activity of membrane proteins, which may serve as specific receptors, pumps, carriers, channels or enzymes, ensures proper functioning of the organelle. Investigations of these proteins should help to answer questions addressing these functions as well as the biogenesis of the membrane.

Characterization of the IMPs necessitated the

isolation of highly purified peroxisomes and subsequent preparation of a membrane fraction. An antiserum was raised against purified peroxisomal membranes which reacted with six peroxisomal IMPs (molecular masses 140 kDa, 69 kDa, 50 kDa, 36 kDa, 22 kDa and 15 kDa). We investigated the response of these IMPs to clofibrate treatment, determined if any of these IMPs co-localize to other subcellular organelles, and determined the cellular site of synthesis of several of these IMPs. The implications of the results for peroxisomal function and biogenesis will be discussed.

## 3.1 Subcellular Fractionation of Rat Liver

### 3.1.1 Isolation of Peroxisomes, Mitochondria and Lysosomes

Subcellular fractionations of rat liver were performed according to the procedure developed by Leighton *et al* (1968) as modified by Alexson *et al* (1985). The isolation requires treatment of the rats with the detergent Triton WR1339 (tyloxapol), which accumulates in the lysosomes causing a decrease in their equilibrium density. This allows for separation of lysosomes from peroxisomes which have overlapping densities in normal rat liver. This procedure was performed on untreated rats and rats treated with the hypolipidemic drug, clofibrate, which causes peroxisomal proliferation and effectively increases peroxisomal content of liver four- to ten-fold (Lazarow and de Duve, 1976). The

fractionation procedure involves differential centrifugation of a rat liver homogenate to generate a fraction rich in peroxisomes, lysosomes and mitochondria followed by isopycnic centrifugation in a sucrose density gradient. The separation procedure is outlined in Fig. 3.1.1. The distribution of cellular organelles in the various fractions was determined by analyses of marker enzymes. The organelles and their respective marker enzymes are as follows: catalase for peroxisomes, cytochrome c oxidase for mitochondria, N-acetyl-ß-glucosaminidase for lysosomes and esterase for microsomes (Hartl *et al*, 1985). The results of the preliminary differential centrifugation steps are summarized in Table 3.1.1A for untreated rats (n=3) and Table 3.1.1B for clofibrate-treated rats (n=5). Generally, the percent distribution of marker enzymes in the heavy mitochondrial ($\nu$), microsomal ($\psi$) and light mitochondrial ($\lambda$) fractions resembled that found by Leighton *et al* (1968). The light mitochondrial ($\lambda$) fraction was further purified by sucrose density gradient centrifugation in order to generate a highly purified peroxisomal fraction. The $\lambda$ fraction contained a high percentage of mitochondria, lysosomes and peroxisomes and had a small amount of microsomal contamination (6.1 ± 1.1% for untreated rats and 5.6 ± 1.1% for clofibrate-treated rats) as indicated by esterase activity (Table 3.1.1A and 3.1.1B). The percent distribution of esterase activity in the $\lambda$ fraction was higher than that found by Leighton *et al* (1968)

Fig. 3.1.1. Flowchart of the Subcellular Fractionation

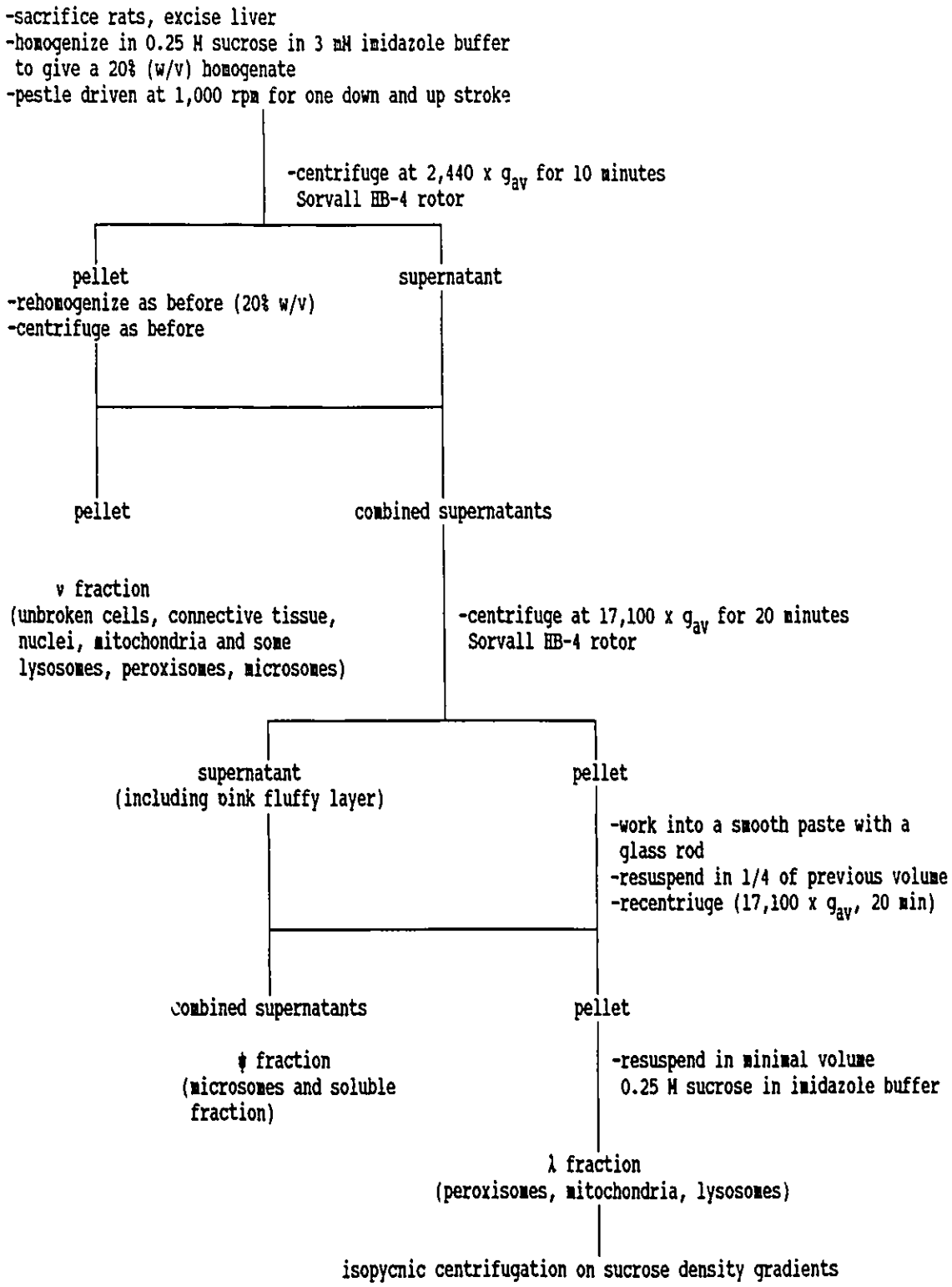


Table 3.1.1A. Primary Fractionation of Liver From Untreated Rats

| Enzyme | Percent distribution: Heavy Mitochondrial fraction ($\nu$) | Microsomal fraction ($\psi$) | Light Mitochondrial fraction ($\lambda$) | Overall Recovery |
|---|---|---|---|---|
| Protein | 27.4 ± 1.2 | 55.5 ± 3.1 | 15.8 ± 3.2 | 98.7 ± 7.5 |
| Catalase | 11.00 ± 0.26 | 37.0 ± 8.0 | 30.6 ± 7.2 | 78.6 ± 15.5 |
| Cytochrome oxidase | 53.4 ± 2.8 | 25.8 ± 1.0 | 25.0 ± 8.3 | 104.2 ± 12.1 |
| Esterase | 17.4 ± 3.6 | 65.6 ± 9.1 | 6.1 ± 1.1 | 89.1 ± 13.8 |
| ß-glucosaminidase | 19.8 ± 0.50 | 31.0 ± 3.0 | 41.8 ± 1.5 | 92.6 ± 5.0 |

Table 3.1.1B. Primary Fractionation of Liver from Clofibrate-Treated Rats

| Enzyme | Percent distribution: Heavy Mitochondrial fraction ($\nu$) | Microsomal fraction ($\psi$) | Light Mitochondrial fraction ($\lambda$) | Overall Recovery |
|---|---|---|---|---|
| Protein | 27.6 ± 5.8 | 67.3 ± 3.2 | 16.8 ± 3.3 | 111.8 ± 12.3 |
| Catalase | 10.1 ± 1.0 | 49.9 ± 4.4 | 18.9 ± 4.5 | 78.9 ± 9.9 |
| Cytochrome oxidase | 41.6 ± 8.5 | 21.7 ± 6.4 | 27.4 ± 5.7 | 90.7 ± 20.6 |
| Esterase | 15.4 ± 2.0 | 60.5 ± 9.0 | 5.6 ± 1.1 | 81.5 ± 12.1 |
| ß-glucosaminidase | 15.6 ± 2.2 | 31.5 ± 3.0 | 37.2 ± 8.5 | 84.3 ± 13.7 |

at 2.4 ± 1.1%. The reason for this is unknown but it was reproducible under our isolation conditions. In addition, the percent distribution of catalase activity in the λ fraction was lower than that of Leighton *et al* (1968). For the untreated rats (Table 3.1.1A) the percent distribution of catalase activity in the λ fraction was 30.6 ± 7.2% compared to 39.0 ± 5.7% found by Leighton *et al* (1968). This was attributed to an increased release of catalase from damaged peroxisomes during our isolation procedure. For clofibrate-treated rats (Table 3.1.1B) the percent distribution of catalase in the λ fraction was only 18.9 ± 4.5% indicating that clofibrate treatment may have caused an increase in the fragility of the peroxisomes, allowing more catalase to leak out during the initial separation steps. This is in agreement with previous studies which indicate that proliferating peroxisomes may have an increased susceptibility to rupture during purification (Hess *et al*, 1965; Klucis *et al*, 1985). It has been suggested that several hypolipidemic drugs, including clofibrate, may exert an effect upon the lability of the membrane, leading to an increased release of peroxisomal enzymes into the cytosol during the isolation procedures or that the drugs may directly effect permeability of the membrane and allow a redistribution of the enzymes between subcellular compartments *in vivo* (Klucis *et al*, 1985; Hemsley *et al*, 1988; Crane *et al*, 1988).

Following isopycnic centrifugation of the λ fraction,

the fractions collected from the sucrose gradient were analyzed for marker enzyme activity. The results are shown in Fig. 3.1.2, panel A for untreated rats and Fig. 3.1.2, panel B for rats treated with clofibrate. The sedimentation patterns of the various organelles in the sucrose density gradients did not show any differences between untreated and clofibrate-treated rats, in agreement with previous studies (Hayashi *et al*, 1975). The peak of peroxisomes was found at an average equilibrium density of 1.23 g/cm$^3$, identical to the literature value (Leighton et al, 1968). There was a secondary peak of catalase activity found at low density which was probably due to the release of this peroxisomal matrix enzyme from disrupted peroxisomes. It has been shown that rat liver peroxisomes are selectively leaky to catalase upon damage incurred during isolation procedures (Alexson *et al*, 1985). Mitochondria had a mean equilibrium density of 1.19 g/cm$^3$, in close agreement with 1.18 g/cm$^3$ reported by Leighton *et al* (1968). The treatment with tyloxapol caused the equilibrium density of lysosomes to decrease from 1.21 g/cm$^3$ (normal density) to 1.10 g/cm$^3$ due to the accumulation of tyloxapol in the lysosomes. The amount of microsomes on the gradient was relatively low; however, there were two small esterase peaks at 1.20 g/cm$^3$ and 1.16 g/cm$^3$. This bimodal distribution pattern in sucrose density gradients is typical of a group of microsomal enzymes including esterase and glucose-6-phosphatase (Beaufay *et al*, 1974b). This

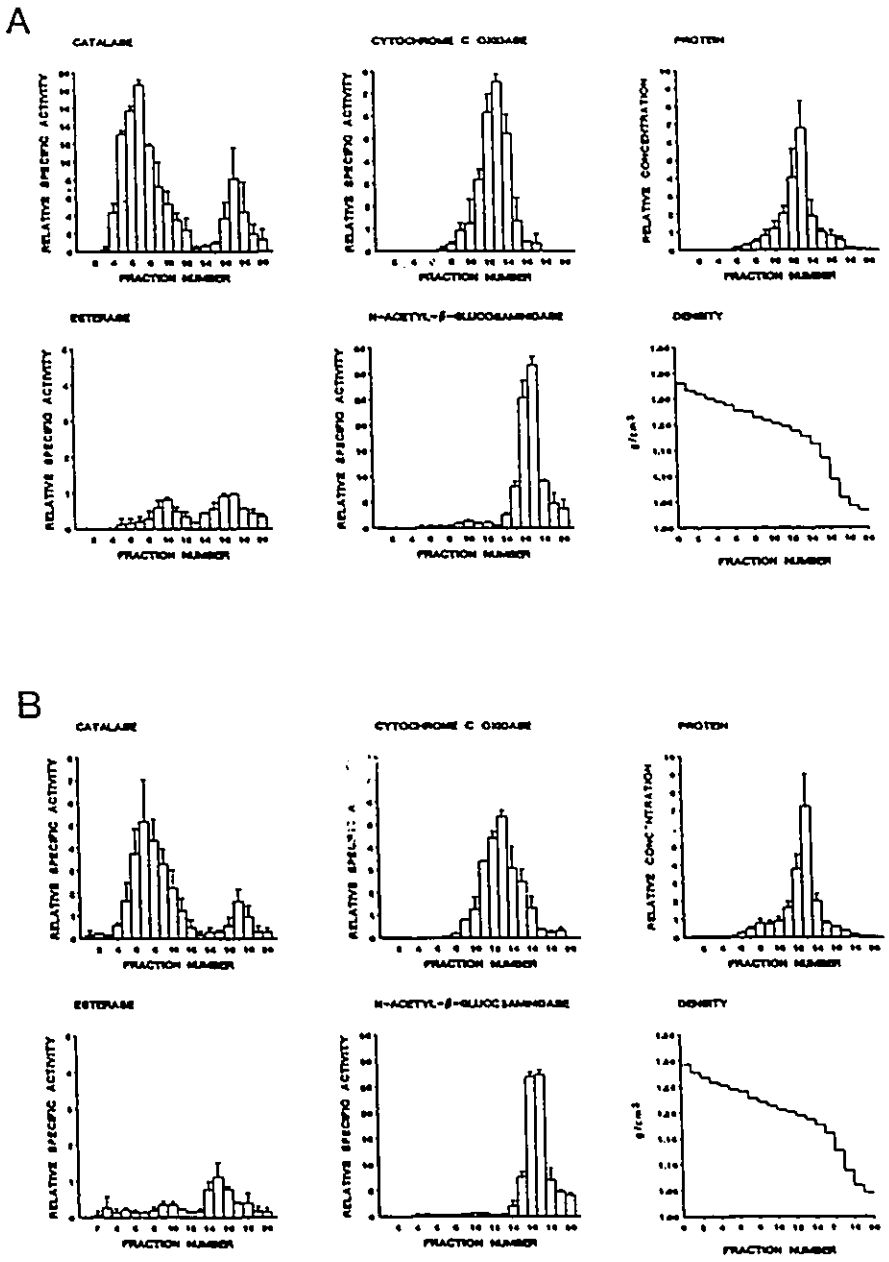



Fig. 3.1.2 Distribution of subcellular organelles from untreated (panel A) and clofibrate-treated (panel B) rat livers following isopycnic centrifugation in sucrose gradients. The organelles and their respective marker enzymes are as follows: peroxisomes-catalase, mitochondria-cytochrome c oxidase, lysosomes- N-acetyl-ß-glucosaminidase, microsomes-esterase. Enzyme levels are recorded as relative specific activities and protein levels as relative concentrations. The direction of decreasing density is from left to right, shown in the bottom right graphs in panels A and B.

distribution may reflect the separation of particles with different densities due to an increase in the number of ribosomes per unit membrane surface area, variations in protein to phospholipid ratios resulting from differences in composition of the vesicles, or both (Beaufay *et al*, 1974b).

The purity of a fraction is indicated by the relative specific activities of marker enzymes in that fraction. The relative specific activity is the specific activity of an enzyme in the individual fraction with respect to the specific activity of that enzyme in the whole liver homogenate. The relative specific activity of catalase in the peak fraction of untreated rats was lower than that previously observed (Leighton *et al*, 1968). However, this was probably due to the large amount of catalase that leaked out of peroxisomes during the initial steps in the separation procedure and during the isopycnic centrifugation step as seen by the rather large secondary peak of catalase (Fig. 3.1.2, panel A). The relative specific activity of catalase in the peroxisomal fraction of clofibrate-treated rats was lower than that for untreated rats. This result does not imply that the clofibrate peroxisomes were impure but is due to the fact that the catalase activity is only slightly increased per gram liver while clofibrate treatment causes a three- to ten- fold increase in the protein content of the peroxisomal fraction (Hartl and Just, 1987). The relative specific activities of the marker enzymes in purified peroxisomal, mitochondrial and

Table 3.1.2A Properties of the Peroxisomal, Mitochondrial and Lysosomal Fractions From Untreated Rats Following Isopycnic Density Gradient Centrifugation.

| | Relative Specific Activity | | |
|---|---|---|---|
| Organelle/ Marker Enzyme | Peroxisomal Fraction | Mitochondrial Fraction | Lysosomal Fraction |
| Peroxisomes/ catalase | 18.63 ± 0.52 | 0.35 ± 0.32 | 8.1 ± 3.4 |
| Mitochondria/ cytochrome oxidase | 0.08 ± 0.12 | 7.53 ± 0.35 | 0.31 ± 0.44 |
| Microsomes/ esterase | 0.19 ± 0.15 | 0.17 ± 0.03 | 0.95 ± 0.05 |
| Lysosomes/ β-glucosaminidase | 0.32 ± 0.25 | 0.52 ± 0.09 | 31.6 ± 1.7 |

Table 3.1.2B Properties of the Peroxisomal, Mitochondrial and Lysosomal Fractions From Clofibrate-Treated Rats Following Isopycnic Density Gradient Centrifugation.

| | Relative Specific Activity | | |
|---|---|---|---|
| Organelle/ Marker Enzyme | Peroxisomal Fraction | Mitochondrial Fraction | Lysosomal Fraction |
| Peroxisomes/ catalase | 5.2 ± 1.9 | 0.15 ± 0.10 | 1.64 ± 0.54 |
| Mitochondria/ cytochrome oxidase | 0.07 ± 0.01 | 5.34 ± 0.29 | 0.34 ± 0.06 |
| Microsomes/ esterase | 0.11 ± 0.04 | 0.16 ± 0.08 | 0.38 ± 0.06 |
| Lysosomes/ β-glucosaminidase | 0.32 ± 0.09 | 0.43 ± 0.11 | 27.31 ± 0.93 |

lysosomal fractions collected from the sucrose gradients are tabulated in Table 3.1.2A for untreated rats and Table 3.1.2B for clofibrate-treated rats.

The percent contamination of the peroxisomal fractions with the other organelles is shown in Table 3.1.3A and 3.1.3B. This was calculated by multiplying the relative specific activity of a marker enzyme in a particular fraction by the percent of total rat liver protein that that particular organelle constitutes (Fujiki *et al* 1982b). The results indicated that the peroxisomes were greater than 90% pure. An estimate of peroxisomal purity based on catalase activity was not possible because it was difficult to compensate for the leakage of catalase from peroxisomes in these calculations (Alexson *et al*, 1985; Klucis *et al*, 1985). The calculations in Table 3.1.3B were performed, assuming that the proportion of rat liver proteins constituted by mitochondria, ER and lysosomes was unchanged by clofibrate treatment. This assumption is consistent with our data, which have shown no changes in the relative specific activities of marker enzymes for these organelles upon clofibrate treatment, and data of Crane *et al* (1985) who reported no significant increase in specific content of these organelles in clofibrate-treated mouse liver. Although some reports have documented clofibrate-induced changes in the other cellular organelles, these levels have not been reproducibly quantitated (Hess *et al*, 1965; Markwell *et al*, 1977; Lipsky and Pedersen, 1982).

Table 3.1.3A. Purity of the Peroxisomal Fractions From Untreated Rats After Isopycnic Density Gradient Centrifugation

| Organelle/ Marker Enzyme | RSA of the Marker Enzyme in the Peroxisome Fraction | X | % Total Liver Protein The Organelle Constitutes[a] | = | % Contamination of the Peroxisomal Fraction |
|---|---|---|---|---|---|
| Mitochondria/ cytochrome oxidase | 0.08 ± 0.12 | X | 20.2% | = | 1.7 ± 2.4% |
| Microsomes/ esterase | 0.19 ± 0.15 | X | 21.5% | = | 4.2 ± 3.2% |
| Lysosomes/ β-glucosaminidase | 0.32 ± 0.25 | X | 2.03% | = | 0.65 ± 0.51% |

[a]Taken from Leighton *et al* (1968).

Table 3.1.3B. Purity of the Peroxisomal Fractions From Clofibrate-Treated Rat Liver After Isopycnic Density Gradient Centrifugation

| Organelle/ Marker Enzyme | RSA of the Marker Enzyme in the Peroxisome Fraction | X | % Total Liver Protein The Organelle Constitutes[a] | = | % Contamination of the Peroxisomal Fraction |
|---|---|---|---|---|---|
| Mitochondria/ cytochrome oxidase | 0.07 ± 0.01 | X | 20.2% | = | 1.41 ± 0.20 |
| Microsomes/ esterase | 0.11 ± 0.04 | X | 21.5% | = | 2.37 ± 0.86 |
| Lysosome/ β-glucosaminidase | 0.32 ± 0.09 | X | 2.03% | = | 0.65 ± 0.18 |

[a]This value assumes that the proportions of rat liver protein constituted by mitochondria, ER and lysosomes were unchanged in clofibrate-treated rat liver.

SDS-PAGE analysis of fractions collected from the sucrose density gradient confirmed that the peroxisomal fractions were highly purified. Fractions from untreated and clofibrate-treated rats are shown in Fig. 3.1.3 panel A and panel B, respectively. The organellar peaks were not as tightly banded in the gradient represented in panel B compared to panel A, but it was still possible to compare the polypeptide patterns of the purest peroxisomal, mitochondrial and lysosomal fractions. In the peak peroxisomal fractions the major peroxisomal polypeptides are clearly visible, including the enzymes involved in ß-oxidation of fatty acids: hydratase dehydrogenase (74 kDa), fatty acyl-CoA oxidase A, B and C subunits (68 kDa, 51 kDa and 23 kDa, respectively) and thiolase (41 kDa), as well as catalase (64 kDa) and urate oxidase (33 kDa) (Fig. 3.1.3, panel C) (Lazarow *et al*, 1982a; 1982b; Goldman and Blobel, 1978). The enzymes involved in ß-oxidation were all induced by treatment with clofibrate as seen in Fig. 3.1.3, panel C. The polypeptide profiles of peak mitochondrial and lysosomal fractions were not greatly effected by clofibrate treatment (Fig. 3.1.3, panel A and B). This is in agreement with the observation that the relative specific activities of cytochrome c oxidase and N-acetyl-ß-glucosaminidase in the mitochondrial and lysosomal peak fractions, respectively, were not effected by clofibrate treatment (Fig. 3.1.2, compare panels A and B). In addition, previous studies have reported that although clofibrate has

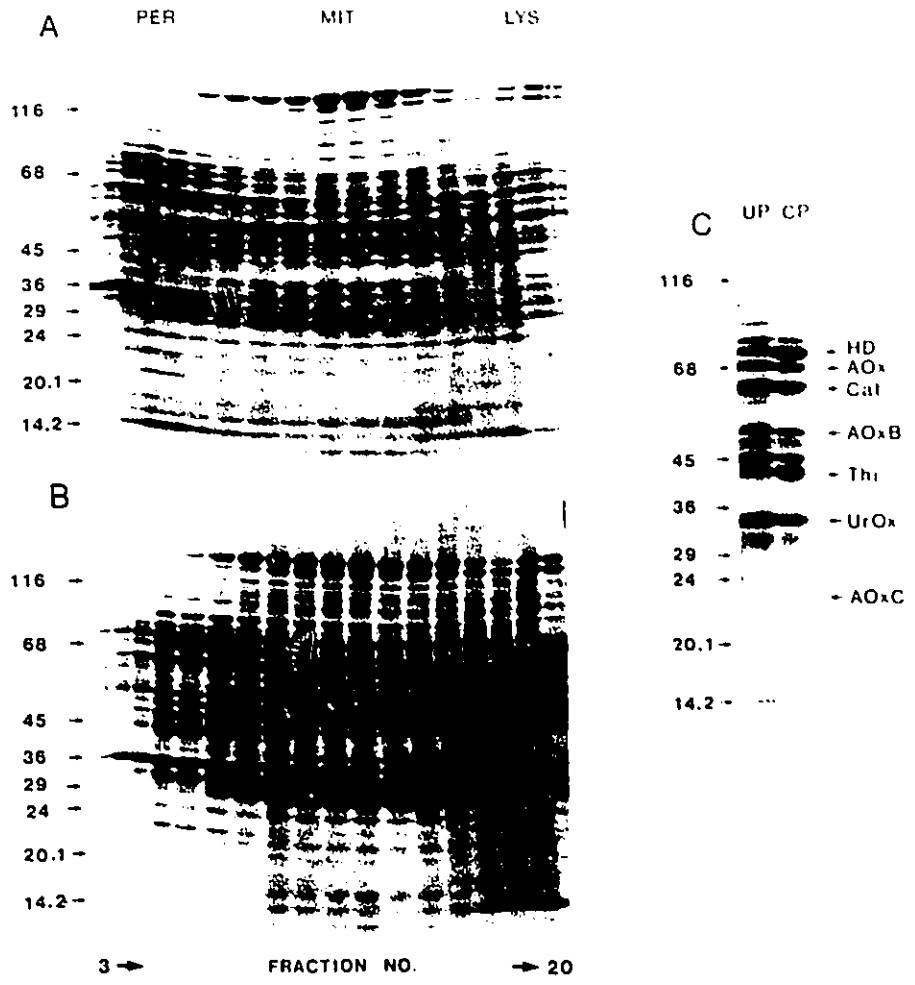



Fig. 3.1.3. SDS-PAGE analysis of subcellular fractions from untreated (panel A) and clofibrate-treated (panel B) rat livers collected from the sucrose density gradients (20 μg protein per lane). The peaks of peroxisomes (PER), mitochondria (MIT) and lysosomes (LYS) are indicated at the top of panel A. The direction of decreasing density is from left to right. Molecular mass protein standards (in kDa) are indicated at the left: *E. coli* ß-galactosidase (116), bovine serum albumin (68), ovalbumin (45), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36), bovine erythrocyte carbonic anhydrase (29), bovine pancreas trypsinogen (24), soybean trypsin inhibitor (20.1) and bovine milk α-lactalbumin (14.2). Panel C compares the peak of peroxisomes from untreated (UP) and clofibrate-treated (CP) rat liver. Some major peroxisomal enzymes are indicated at the right: HD- hydratase dehydrogenase, AOx-fatty acyl-CoA oxidase, AOxB- B subunit of AOx, AOxC- C subunit of AOx, Cat- catalase, Thi- thiolase, UrOx- urate oxidase.

effects on a few specific mitochondrial enzymes involved in lipid metabolism, the SDS polyacrylamide gel patterns of mitochondria from control and treated rat livers are very similar (Lipsky and Perdersen, 1982).

### 3.1.2 Isolation of Rat Liver Microsomes

Rat liver microsomes were isolated from clofibrate-treated Sprague-Dawley rats by the differential centrifugation and equilibrium density centrifugation procedure of Bergeron (1979) as modified by Paiement and Bergeron (1983). The resultant microsomal pellet, enriched for rough microsomes, was assayed for marker enzyme activities. The purity of the fraction is indicated in Table 3.1.4. The values determining purity were calculated using the proportion of liver protein constituted by the various organelles in untreated rat liver. The peroxisomal value was corrected for, because it is well documented that clofibrate treatment causes a 4- to 10-fold increase in peroxisomes in hepatocytes (Lazarow and de Duve, 1976). The effects on the other organelles under these conditions are not well characterized; however, Crane *et al* (1985) reported no significant increase in specific content of mitochondria, ER and lysosomes in clofibrate-treated mouse liver. This is consistent with our data in rat liver, which indicated no changes in the relative specific activities of marker enzymes for these organelles upon clofibrate treatment.

Table 3.1.4 Purity of the Rat Liver Microsomal Fraction

| Organelle/ Marker Enzyme | RSA of the Marker Enzyme in the Microsomal Fraction | X | % Total Liver Protein the Organelle Constitiutes | = | % Contamination of the Microsomal Fraction |
|---|---|---|---|---|---|
| Peroxisomes/ catalase | 0.066 | X | 2.53% (10.12-25.3%)[a] | = | 0.17% (0.67-1.7%) |
| Mitochondria/ cytochrome oxidase | 0.18 | X | 20.2%[b] | = | 3.6% |
| Lysosomes/ β-glucosaminidase | 0.24 | X | 2.03%[b] | = | 0.49% |
| Microsomes/ esterase | 6.2 | X | 21.5%[b] | = | 133.3% |

[a]The values in brackets are corrected for the 4- to 10-fold increase in peroxisomal content in clofibrate-treated rat liver (Lazarow and de Duve, 1976).

[b]These values assume that the proportion of rat liver protein constituted by mitochondria, lysosomes and ER are unchanged in clofibrate-treated rat liver.

## 3.2 Isolation of Peroxisomal Membranes

The purest peroxisomal fractions, taken from the dense side of the peak of catalase activity, were used to prepare peroxisomal membranes by the $Na_2CO_3$ procedure of Fujiki *et al* (1982a). This procedure converts closed vesicles to open membrane sheets containing only IMPs, releasing matrix and peripheral membrane proteins (Fujiki *et al*, 1982a). The polypeptide composition of the membrane fractions and the soluble fractions were compared to total peroxisomes using SDS-PAGE analysis (Fig. 3.2.1). Proteins in the soluble fraction were not detected in the peroxisomal membrane fraction (Fig. 3.2.1, compare lanes PS and PM). These included the matrix enzymes involved in ß-oxidation (hydratase dehydrogenase, acyl-CoA oxidase and thiolase) as well as catalase and urate oxidase. On occasion, urate oxidase was found contaminating the membrane preparations. Urate oxidase is a component of the paracrystalline core of rat liver peroxisomes and has been shown to co-purify with peroxisomal membranes in previous studies (Hardeman *et al*, 1990). Washing the membrane preparation copiously with $H_2O$ usually solved this problem.

SDS-PAGE analyses of the peroxisomal membranes revealed several prominent IMPs with molecular masses of approximately 69 kDa, 22 kDa and 15 kDa as well as several other IMPs (75 kDa, 50 kDa, 36 kDa, 32 kDa, 30 kDa, 28 kDa and 24 kDa) (Fig. 3.2.1, lane PM). The patterns for untreated and clofibrate-

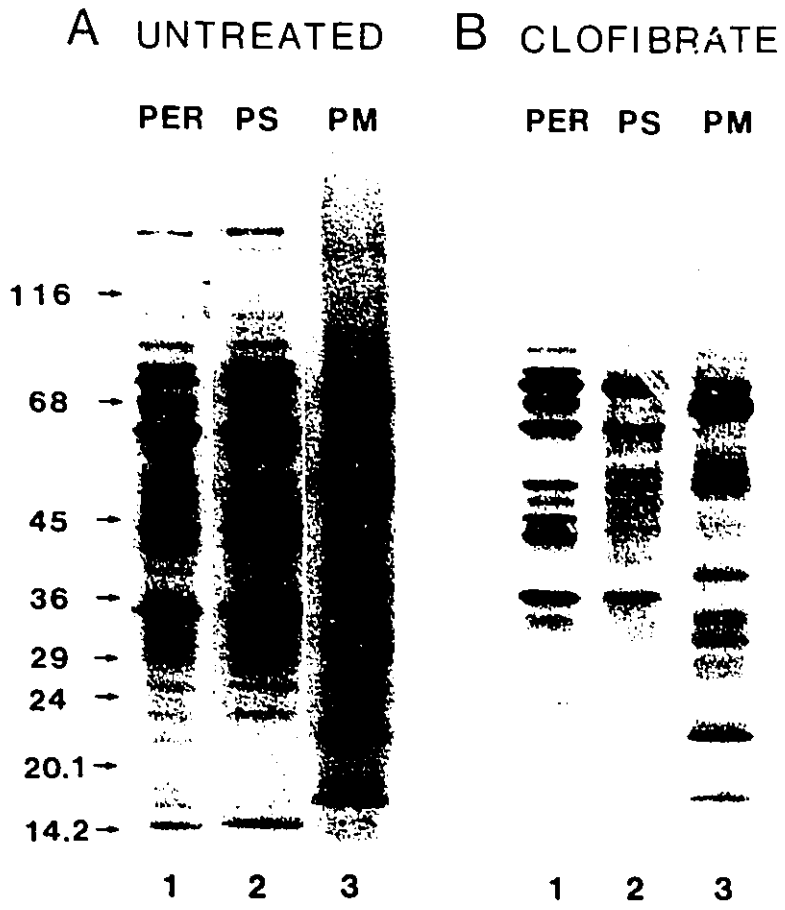



Fig. 3.2.1. SDS-PAGE analysis of rat liver peroxisomes and peroxisomal membranes isolated from untreated (panel A) and clofibrate-treated (panel B) rats. Lane 1, rat liver peroxisomes (20 μg) (PER); lane 2, soluble proteins of peroxisomes released during the preparation of membranes (20 μg) (PS); lane 3, integral membrane polypeptides of rat liver peroxisomes (40 μg) (PM). Molecular mass protein standards (in kDa) are shown at the left.

treated rat liver peroxisomal IMPs closely resemble published patterns for both untreated and clofibrate-treated rats (Fujiki *et al*, 1982b; Köster *et al*, 1986; Hartl and Just, 1987). Although the major 22 kDa IMP was unaffected by clofibrate treatment, the 69 kDa IMP was noticeably induced and the other IMPs (50 kDa, 36 kDa, 32 kDa, 30 kDa, 28 kDa and 15 kDa) were affected to a lesser extent (discussed in section 3.4).

The peroxisomal membrane preparations contained 9.03 ± 2.00% (n=3) and 11.53 ± 1.19% (n=3) of total peroxisomal protein in untreated rats and clofibrate-treated rats, respectively. These values compare with results reported for both normal rat liver peroxisomes (10.59% and 12% reported by Hartl and Just, 1987 and Fujiki *et al*, 1982b, respectively) and peroxisomes isolated from rat livers treated with clofibrate (14.08%) (Hartl and Just, 1987).

## 3.3 Characterization of Anti-Peroxisomal IMP Serum

### 3.3.1 Immunoblot Analyses of Rat Liver Subcellular Fractions With Anti-Peroxisomal IMP Serum

The peroxisomal membrane preparations were used to raise an anti-IMP serum by injections into the popliteal lymph nodes of rabbits (Goudie et al, 1966). Immunoblot analyses with this antiserum showed a reaction with six peroxisomal IMPs of molecular masses 140 kDa, 69 kDa, 50 kDa, 36 kDa, 22

kDa and 15 kDa (Fig. 3.3.1, panel B, lane PM). The antiserum also reacted with a peroxisomal matrix polypeptide of molecular mass 33 kDa (Fig. 3.3.1, panel B, lane PER), which often co-purified with the peroxisomal membranes and based on its molecular mass and relative abundance is thought to be urate oxidase.

To determine if these IMPs were located exclusively in peroxisomes or whether they occurred in other subcellular organelles, immunoblots were performed on purified organelles and $Na_2CO_3$ extracted membranes of those organelles (Fig. 3.3.1). Peroxisomes, mitochondria and lysosomes were isolated from the sucrose gradient (Leighton et al, 1968), and a highly purified microsomal fraction was isolated from clofibrate-treated rats by the procedure of Bergeron (1979) as modified by Paiement and Bergeron (1983). The anti-IMP serum reacted with a 15 kDa polypeptide present in the membranes of all subcellular fractions. This is probably the same 15 kDa IMP that has been previously identified in the membranes of rat liver subcellular organelles by SDS-PAGE analysis (Fujiki et al, 1982b). With the exception of the 15 kDa IMP, there was no significant reaction of the antiserum with mitochondrial or lysosomal polypeptides (Fig. 3.3.1, panel B, lane MIT, MM, LYS and LM). The band which reacted at 33 kDa (Lanes MIT and LYS) was the result of a small amount of contamination of the lysosomal and mitochondrial fractions with peroxisomes. The antiserum reacted with two IMPs of the microsomal membrane

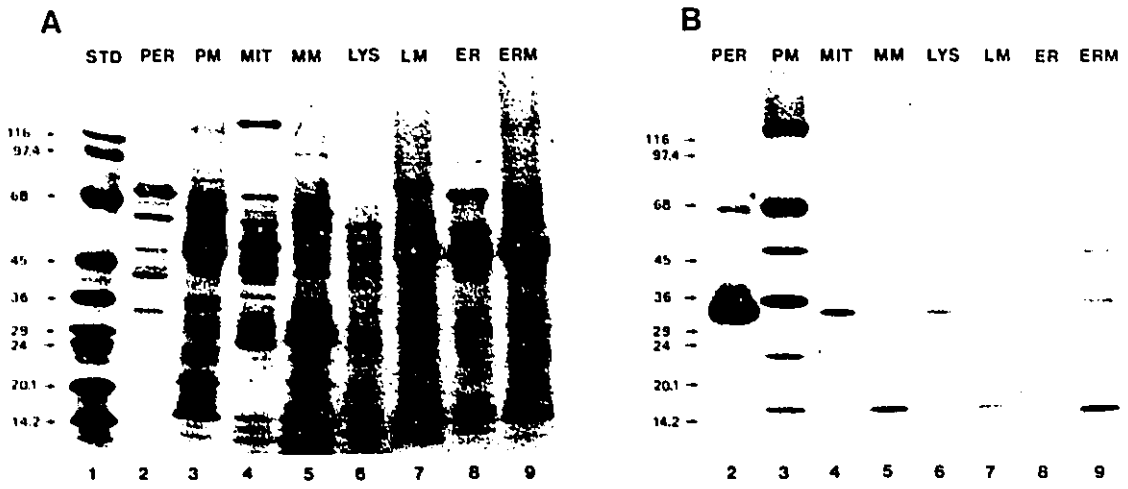



Fig 3.3.1. Immunoblot analysis of rat liver subcellular fractions with anti-peroxisomal IMP serum. Purified peroxisomal, mitochondrial, lysosomal and microsomal fractions (20 $\mu$g) and their $Na_2CO_3$ extracted membranes (50 $\mu$g) were separated by SDS-PAGE prior to transfer. Panel A: SDS-polyacrylamide gel stained with Coomassie Brilliant Blue. Panel B: Corresponding immunoblot. Molecular mass standards are indicated at the left (in kDa). Lane 1, protein standards; lane 2, peroxisomes (PER); lane 3, peroxisomal membranes (PM); lane 4, mitochondria (MIT); lane 5, mitochondrial membranes (MM); lane 6, lysosomes (LYS); lane 7, lysosomal membranes (LM); lane 8, microsomes (ER); lane 9, microsomal membrane (ERM).

which co-migrated with the 50 kDa and 36 kDa IMPs of the peroxisome (Fig. 3.3.1, panel B, lane ERM). Densitometric analysis of the immunoblot showed that there were approximately five times more 50 kDa IMP and eight times more 36 kDa IMP in peroxisomal membranes as compared with microsomal membranes (based on equal amounts of protein loaded on the gel). It is doubtful that the reaction of the antiserum with 50 kDa and 36 kDa IMPs of the microsomal membrane was due to contamination of the microsomal fraction with peroxisomes, since the microsomal fraction was only 0.67-1.7% contaminated with peroxisomes (determined by the relative specific activity of catalase in the ER fraction, Table 3.1.4) and the 140 kDa, 69 kDa and 22 kDa IMPs were absent from the microsomal membrane fraction (Fig. 3.3.1, panel B, lane ERM). Conversely, the 36 kDa and 50 kDa IMPs in the peroxisomal membrane fraction (Fig. 3.3.1, panel B, lane PM) were not due to contamination of the peroxisomal membrane fraction with microsomal membranes, as the ratios of intensities of the 36 kDa and the 50 kDa IMPs to the 15 kDa IMP in the peroxisomal membrane fraction (Fig. 3.3.1, panel B, lane PM) were reversed in the ER membrane fraction (Fig. 3.3.1, panel B, lane ERM). If the 36 kDa and 50 kDa IMPs in the peroxisomal membrane fraction were due to contamination with ER membranes, the ratios of the intensities of the 36 kDa and the 50 kDa IMPs to the intensity of the 15 kDa IMP should have remained the same for both the peroxisomal membrane and

the ER membrane fractions (or even decreased in the peroxisomal membrane fraction due to the presence of 15 kDa IMP native to the peroxisomal membrane). This result demonstrates that the 36 kDa and 50 kDa IMPs are true IMPs of the peroxisomal membrane and not the result of spurious contamination by ER membrane IMPs. Therefore it would appear that these immunoreactive IMPs (36 kDa and 50 kDa) are co-localized to peroxisomes and ER. Hartl and Just (1987) have also found that antiserum raised against the 36 kDa IMP of peroxisomes reacts with a protein of the same molecular mass in microsomal membranes.

To verify further the identity of the IMPs, immunoblot analyses using the anti-IMP serum were performed across all the fractions collected from the sucrose gradient (Fig. 3.3.2). As controls, the distribution of catalase activity (relative specific activity) was measured across the gradient, and the immunoblots were performed with antiserum against cytochrome P-450 PB-1, an enzyme of the smooth ER, and anti-ribophorin I, enriched primarily in the rough ER (Pugsley 1989). The peak of catalase activity was found in fraction 7 (1.24 $g/cm^3$). Both microsomal markers exhibited bimodal distributions with peaks in fraction 10 (1.20 $g/cm^3$) and fraction 15 (1.16 $g/cm^3$). The distributions of the 69 and 22 kDa IMPs paralleled that of peroxisomal catalase. The 15 kDa IMP was distributed across the entire gradient and the distribution of the 36 kDa IMP could not be determined,

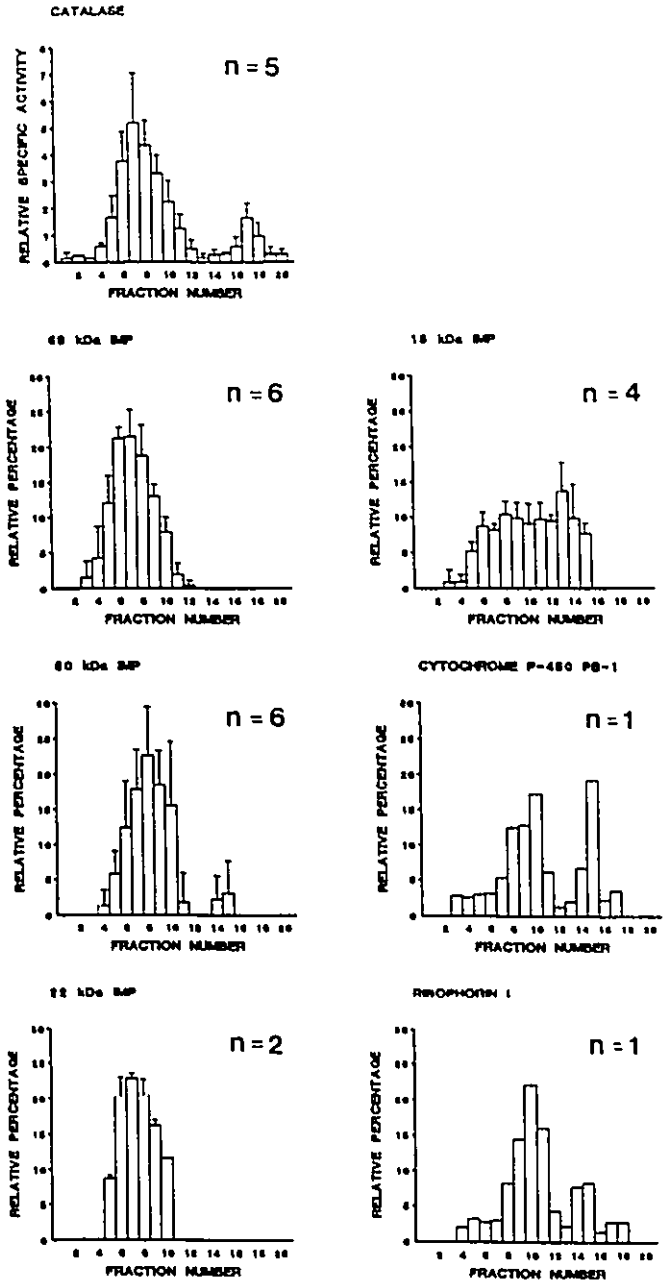



Fig. 3.3.2. Distribution of catalase, IMPs and microsomal markers across the sucrose density gradient. Catalase was measured as relative specific activity. The distribution of the IMPs and microsomal markers were determined by densitometric analysis of immunoblots. Fractions collected from the sucrose gradients (20 μg per lane) were separated by SDS-PAGE prior to transfer to nitrocellulose. Immunoblot analyses were performed using anti-IMP serum, anti-cytochrome P-450 PB-1 or anti-ribophorin I. The distributions across the gradient were determined by densitometric analysis and plotted as relative percentage of signal in each fraction.

because its signal was masked by a large signal from reaction of the antiserum with urate oxidase (33 kDa). The majority of the signal from the 50 kDa IMP was present in the peroxisomal fraction; however, the peak of 50 kDa was shifted to fraction 8 and a small secondary peak was present in fraction 15. The skewed distribution of the major 50 kDa IMP peak was probably the result of contribution from its presence in microsomal fractions (fraction 10), and the bimodal distribution once again indicated that 50 kDa IMP is a component of both the peroxisomal and ER fractions.

### 3.3.2 Purification of Monospecific IMP Antibodies From Heterogeneous Antiserum

In order to determine if any of the peroxisomal IMPs to which antibodies were raised were related, each component of the heterogeneous anti-IMP serum was affinity purified (Hall *et al*, 1984) and used in immunoblot analyses. Monospecific antibodies to the 15 kDa, 22 kDa, 36 kDa and 50 kDa IMPs were successfully purified from the heterogeneous IMP antiserum (Fig. 3.3.3, lanes 2 to 5) indicating that each of these IMPs is immunologically unrelated. Antibodies against the 69 kDa IMP cross-reacted with the 140 kDa IMP (Fig. 3.3.3, lane 6). Conversely, antibodies against the 140 kDa IMP cross-reacted with the 69 kDa IMP (data not shown) indicating that these two IMPs share antigenic determinants and that the 140 kDa IMP may be a dimer composed of two 69 kDa subunits.

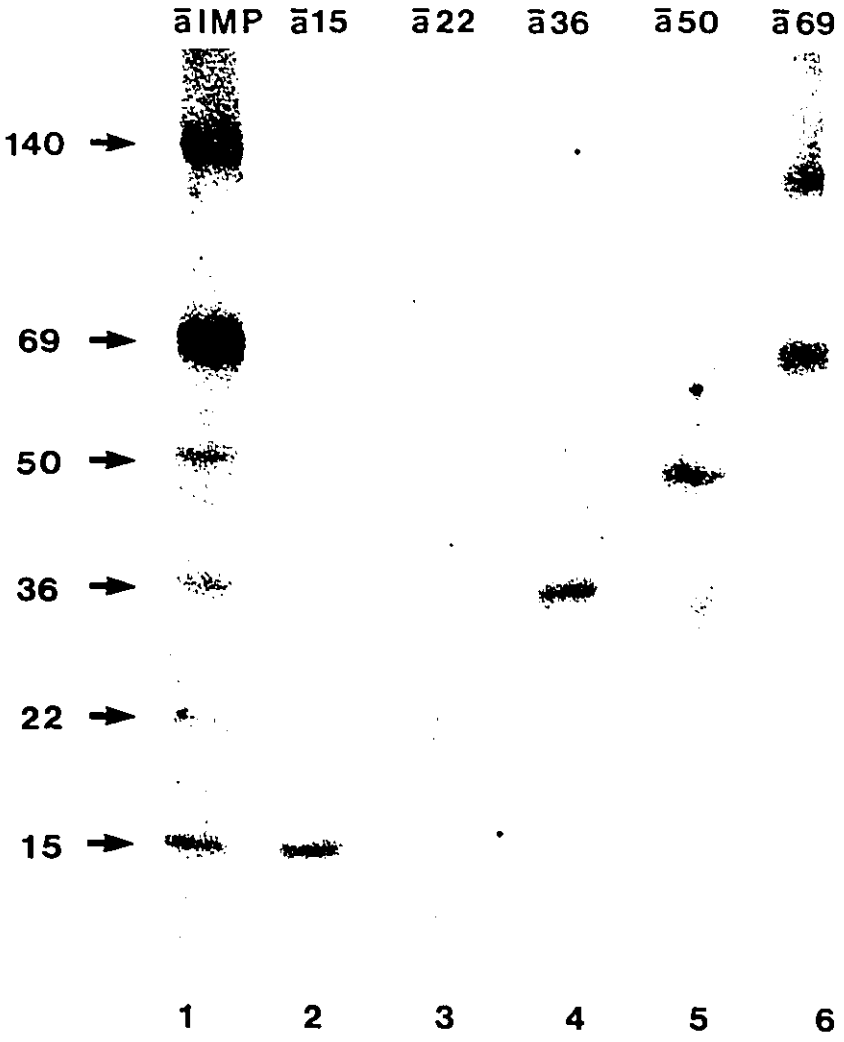



Fig. 3.3.3. Immunoblot analysis using monospecific antibodies purified from heterogeneous anti-peroxisomal IMP serum. Peroxisomal membranes (20 μg) containing IMPs were separated by SDS-PAGE and transferred to nitrocellulose. Lane 1, blot treated with anti-peroxisomal IMP serum; lanes 2-6, blots treated with affinity purified, monospecific antibodies to the 15 kDa IMP, 22 kDa IMP, 36 kDa IMP, 50 kDa IMP and 69 kDa IMP, respectively. The molecular masses of each IMP (in kDa) are indicated at the left.

Santos *et al* (1988a; 1988b) reported that affinity-purified antibodies against the 69 kDa rat liver peroxisomal IMP also recognize the 140 kDa peroxisomal IMP in both rat liver and human fibroblasts.

### 3.3.3 Immunofluorescence Localization of Peroxisomal IMPs in CV-1 Cells

Monospecific antibodies to the 22 kDa, 36 kDa and 50 kDa IMPs, purified from the heterogeneous IMP antiserum, were used for immunofluorescence microscopy using monkey kidney CV-1 cells. The immunofluorescence microscopy was carried out by Dr. Gilbert-André Keller, in the laboratory of Dr. John Singer, in the Department of Biology, University of California at San Diego. Each monospecific antiserum exhibited the characteristic punctate pattern of peroxisomes (Fig. 3.3.4, panel A, B and C). This pattern was similar to that observed upon treatment of CV-1 cells with antibodies against the peroxisomal matrix enzyme catalase (Fig. 3.3.4, panel D), and quite distinct from the lacy reticular pattern obtained when CV-1 cells are stained for ER (Fig. 3.3.4, panel E) (Terasaki *et al*, 1984). These results confirmed that the 22 kDa, 36 kDa and 50 kDa IMPs were primarily located in peroxisomes or an organelle that generates a similar punctate pattern. The apparent lack of labeling of the ER with the monospecific antiserum to the 36 kDa and 50 kDa IMPs was probably due to the low amounts of these polypeptides in the ER membrane,

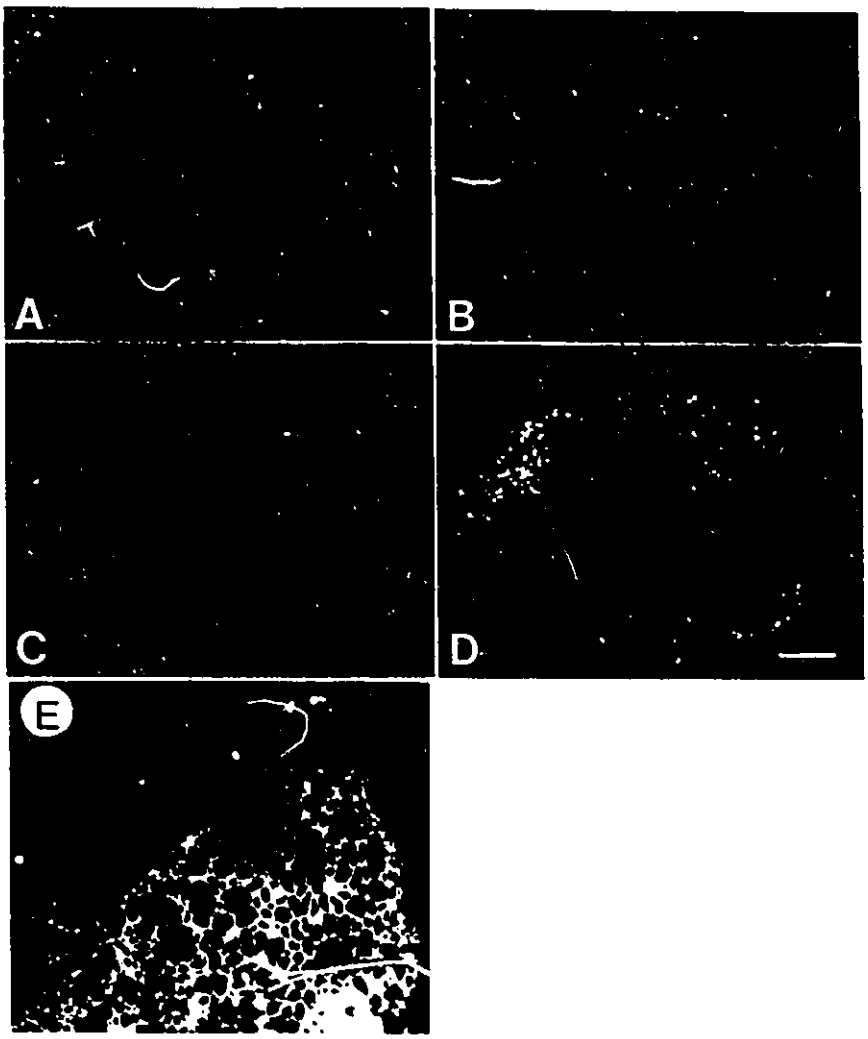



Fig. 3.3.4. Immunofluorescence localization of peroxisomal proteins in CV-1 cells. The respective antibody treatments are as follows: panel A, monospecific antibodies to the 22 kDa IMP; panel B, monospecific antibodies to the 36 kDa IMP; panel C, monospecific antibodies to the 50 kDa IMP; panel D, antibodies to bovine catalase; panel E, CV-1 cell stained with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$),indicating endoplasmic reticulum (taken from Terasaki *et al*, 1984). The bar represents 10 μm in panel A to D and 7.5 μm in panel E.

resulting perhaps from a rapid turnover of these polypeptides in the ER or their exit to another intracellular compartment. Immunofluorescence microscopy using human fibroblasts treated with a polyspecific antiserum detecting peroxisomal IMPs (molecular masses 140 kDa, 69 kDa and 53 kDa, which correspond to the 140 kDa, 69 kDa and 50 kDa IMPs reported here) exhibited a punctate pattern throughout the cytoplasm (Santos *et al*, 1988b). This pattern was similar to that observed when CV-1 cells were treated with monospecific antibodies to the 22 kDa, 36 kDa and 50 kDa IMPs and indicated that antibodies against the 50 kDa IMP do not detect ER membranes in human fibroblasts as well as in CV-1 cells.

Without double-labeling of the CV-1 cells with another peroxisomal antibody it is difficult to conclude that these IMPs (22 kDa, 36 kDa and 50 kDa) are localized to peroxisomes. Through immunoblot analyses we know that these IMPs were not localized to lysosomes or mitochondria; however, they could be localized to some uncharacterized vesicle or perhaps microperoxisomes, generating a punctate pattern upon immunofluorescence microscopy. Microperoxisomes have been characterized as small, elongate or spherical peroxisomes (0.1-1.0 $\mu$m in diameter) which contain only a subset of peroxisomal enzymes (Novikoff *et al*, 1973). Microperoxisomes are present in many mammalian cell types including rat and human hepatocytes and may represent a progenitor of mature peroxisomes or form a distinct class of organelles with a

specialized function (Novikoff *et al*, 1973). Further experimentation involving double-labeling immunofluorescence or immunoelectron microscopy will help to confirm the subcellular location of the IMPs.

### 3.3.4 Reaction of The Anti-Rat Peroxisomal IMP Serum With Human Liver and Yeast Proteins

The reaction of the anti-rat peroxisomal IMP serum with human liver and yeast fractions was investigated by immunoblot analyses. A post-nuclear supernatant of human liver was prepared in the same manner as that of rat liver (Materials and Methods section 2.2.1). This fraction was treated with sodium carbonate generating a human liver membrane fraction. The anti-rat peroxisomal IMP serum reacted with a number of human liver proteins (approximate molecular masses 65, 38, 30, 28, 24, 22 and 14 kDa) (Fig. 3.3.5, panel B, lane 3) but of these, only a 22 kDa polypeptide was integral to a membrane (Fig. 3.3.5, panel B, lane 4). Upon longer exposure there was some reaction of the antiserum with a 36 kDa IMP of human liver. To determine conclusively which component of the polyspecific antiserum was responsible for the cross-reaction, the human liver membrane fractions were treated with affinity purified, mono-specific antibodies to each rat liver peroxisomal IMP. The results indicated that the affinity purified anti-22 kDa IMP serum cross-reacted with a protein of the same molecular weight in the human liver

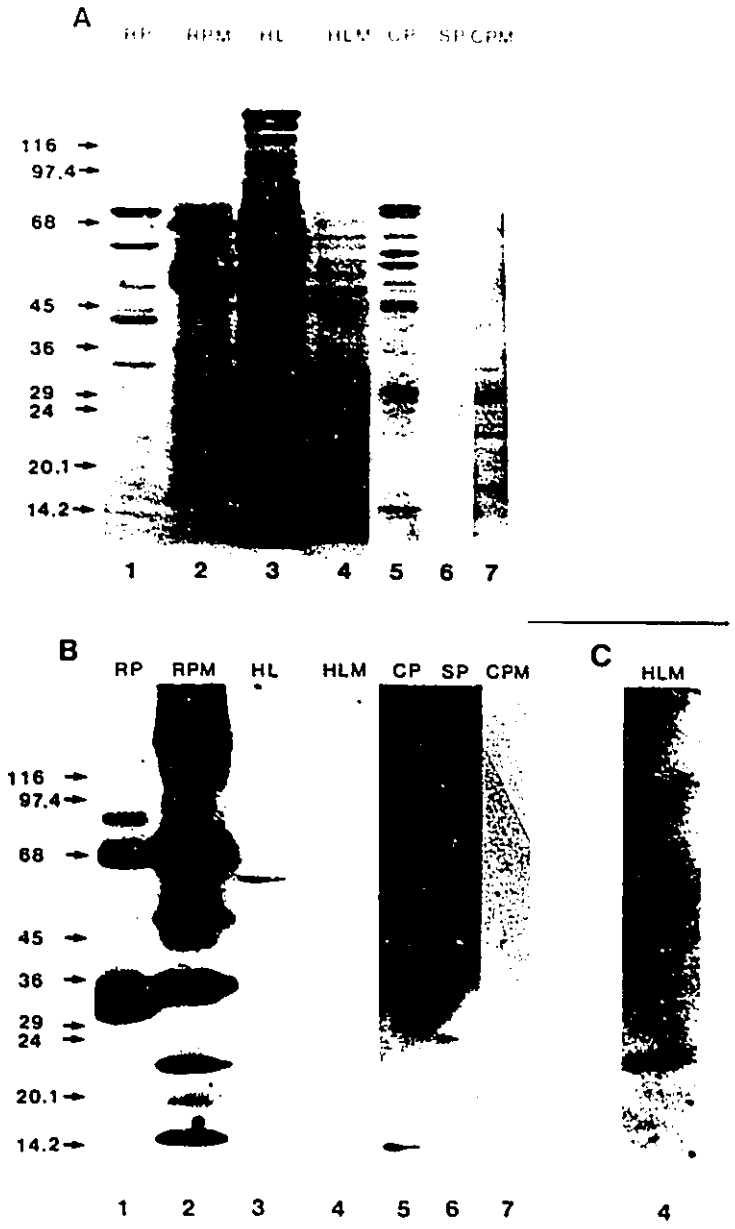



Fig. 3.3.5. Immunoblot of human liver and yeast fractions with anti-rat peroxisomal IMP serum. Panel A, SDS-polyacrylamide gel stained with Coomassie Brilliant Blue; Panel B, corresponding immunoblot; lane 1, rat liver peroxisomes (RP) (20 μg); lane 2, rat liver peroxisomal membranes (RPM) (50 μg); lane 3, human liver post-nuclear supernatant (HL) (300 μg); lane 4, human liver membrane fraction extracted from the PNS by $Na_2CO_3$ treatment (HLM) (50 μg), lane 5, *C. tropicalis* peroxisomes (CP) (50 μg); lane 6, *S. cerevisiae* peroxisomes (SP) (7 μg), Lane 7, membranes isolated from *C.tropicalis* peroxisomes (CPM) (15 μg). Panel C: longer exposure of lane 4, showing a slight cross reaction of anti-IMP serum with the 36 kDa IMP.

membrane fraction (Fig. 3.3.6). The 22 kDa IMP is located exclusively in peroxisomes in rat liver, which leads to the suggestion that this protein may also be peroxisomal in human liver. However, peroxisomes were not isolated from human liver, therefore we cannot rule out the possibility that the 22 kDa IMP may be present in other cellular membranes in human tissues.

Due to similarities in size, extraction properties and antigenicity, one might postulate that the 22 kDa IMPs may perform similar functions in their respective species. The rat liver 22 kDa IMP may be the constituent of the pore in the peroxisomal membrane which confers the unusual permeability properties on this organelle (van Veldhoven *et al*, 1987). Recent evidence indicates that when a rat liver peroxisomal membrane protein fraction containing the 22 kDa IMP was incorporated into liposomes, they were permeable to [$^{14}$C]sucrose (van Veldhoven *et al*, 1987). The high level of permeability of rat peroxisomes for water, cofactors and other solutes the size of sucrose may be a property common to mammals, and therefore it would not be surprising that this pore-forming polypeptide is highly conserved. There is a well characterized class of membrane pore-forming proteins, porins, found in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts which allow diffusion of solutes of molecular weight 4,000-6,000 across these membranes (van Veldhoven *et al*, 1987). To investigate whether the putative

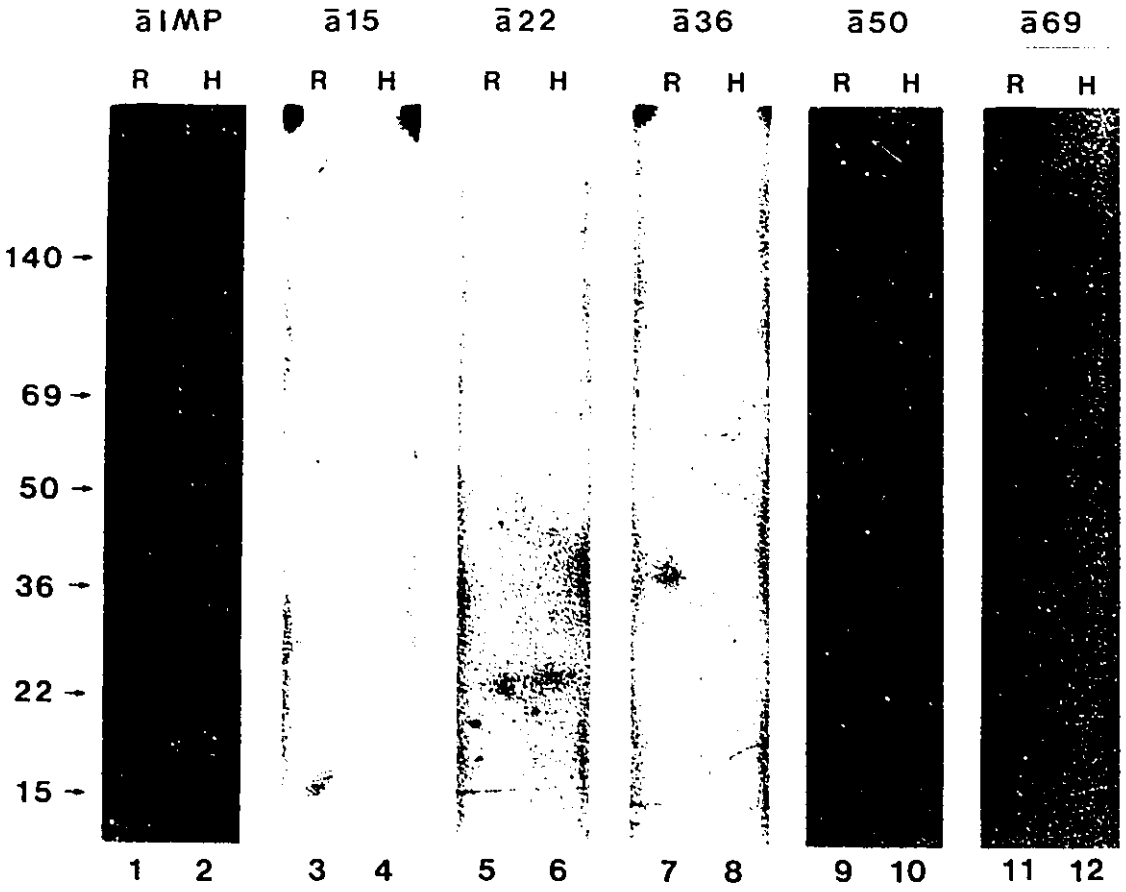



Fig. 3.3.6. Immunoblots of the human liver membrane fractions with affinity-purified, monospecific antibodies to each rat liver peroxisomal IMP. Rat liver peroxisomal membranes (50 μg) were run in the odd-numbered lanes and human liver membranes (600 μg) were run in the even-numbered lanes. The immunoblots were treated with anti-IMP serum (lanes 1 and 2), or monospecific antibodies to the 15 kDa, 22 kDa, 36 kDa, 50 kDa and 69 kDa IMPs in the adjacent panels, labeled above (lanes 3-12). The molecular masses of each IMP (in kDa) are indicated at the left.

pore-forming protein in peroxisomal membranes shared antigenic determinants with porin, an immunoblot was performed with purified peroxisomal membranes from rat liver and a total membrane fraction prepared from the human liver PNS using anti-*Neurospora crassa* porin serum. This antiserum did not recognize the 22 kDa IMP in rat peroxisomes or human liver membranes (data not shown). It did however react with a 30 kDa IMP present in human liver which is probably human mitochondrial porin (data not shown).

The reaction of the anti-rat peroxisomal IMP serum with purified peroxisomes from the yeasts *C. tropicalis* and *S. cerevisiae* (prepared by William Nuttley and John Glover, respectively) was also investigated. There was some reaction of the antiserum with both yeast peroxisomal fractions. *C. tropicalis* peroxisomes exhibited three reacting polypeptides (molecular masses 75 kDa, 43 kDa and <15 kDa), but there was no reaction with IMPs of *C. tropicalis* peroxisomes (*C. tropicalis* peroxisomal membranes were prepared by R.A. Rachubinski) (Fig. 3.3.5 panel B, lanes 5 and 7). There was a reaction of the anti-rat peroxisomal IMP serum with a 24 kDa polypeptide of *S. cerevisiae* peroxisomes (Fig. 3.3.5, panel B, lane 6). Coincidently, there is a major peroxisomal IMP in *S. cerevisiae* at this molecular weight (McCammon *et al.* 1990); however, it was not determined whether this immunoreactive polypeptide was integral to the peroxisomal membrane. In addition, it was not determined which component of the

polyspecific antiserum reacted with this polypeptide of *S. cerevisiae*. Based on its mass, we may speculate that it may share antigenic determinants with the pore-forming peroxisomal 22 kDa IMP. If true, this protein would exhibit evolutionary conservation from yeast to man; however, further experimentation is needed to draw such a conclusion.

### 3.4 Effect of Clofibrate on the Peroxisomal IMPs

Clofibrate is a drug which is widely used in the treatment of human hyperlipidemias (Lazarow and de Duve, 1976). Treatment of rodents with this drug has been shown to induce hepatomegaly and a pronounced proliferation of hepatic peroxisomes, a property which has made clofibrate a useful tool for studying peroxisome biogenesis, function and turnover (Hess *et al*, 1965).

Morphologically, treatment of rodents with clofibrate results in a dramatic proliferation of peroxisomes as well as a moderate increase in smooth ER and mitochondria (Hess *et al*, 1965; Markwell *et al*, 1977; Lipsky and Pedersen, 1982). Previous studies have reported that acid phosphatase (lysosomal marker), glucose-6-phosphatase (ER marker) and cytochrome oxidase (mitochondrial marker) levels are not elevated in liver homogenates of clofibrate-treated rats (Svoboda *et al*, 1967; Cohen and Grasso, 1981; Lazarow and de Duve, 1976). However, activities of enzymes involved in fatty acid oxidation are markedly increased. Treatment with

clofibrate causes an 11- to 18-fold increase in the fatty acid ß-oxidation capacity of liver peroxisomes (Lazarow, 1977) with a concomitant increase in the amounts of the peroxisomal enzymes involved in this process (fatty acyl CoA oxidase, hydratase dehydrogenase and thiolase) (Lazarow *et al*, 1982a). Recently, it has been demonstrated that clofibrate causes an approximately 10-fold increase in microsomal fatty acid hydroxylase activity, accompanied by a similar 10-fold increase in the amount of cytochrome P-452, the isoenzyme responsible for this activity (Sharma *et al*, 1988a; 1988b). Studies on the effects of clofibrate on mitochondrial enzymes have shown that the specific activities of enzymes such as glycerol-1-phosphate dehydrogenase and carnitine acyl transferase increase 6-fold and 10-fold respectively, upon treatment (Hess *et al*, 1965; Markwell *et al*, 1977). Although the induction of peroxisomal, microsomal and mitochondrial enzymes following hypolipidemic challenge appears to be closely linked, the mechanisms underlying these changes in lipid metabolizing enzymes remains to be elucidated (Lock *et al*, 1989). Nevertheless, clofibrate has been a useful experimental tool in peroxisomal research.

To determine the effect of clofibrate-treatment on the IMPs, SDS-PAGE and immunoblots using anti-IMP serum were performed on membranes isolated from liver peroxisomes of untreated and clofibrate-treated rats. The 69 kDa IMP was significantly increased, 6.66 ± 3.05-fold upon clofibrate-

treatment (Fig. 3.4.1, panels A and B, compare lanes 1 and 2 to lanes 3 and 4), which is comparable to that observed by Hartl and Just (1987) who reported a 6-fold increase in the 69 kDa IMP under similar conditions. The large variability in the amount of induction may reflect differences in parameters such as age or hormonal status of the animals employed in the individual experiments (Svoboda *et al*, 1969). The 50 kDa IMP increased to a lesser extent, 2.93 ± 1.18-fold, while the 36 kDa and the 22 kDa IMPs remained unchanged (0.80 ± 0.19-fold and 0.92 ± 0.11-fold, respectively), and the amount of 15 kDa IMP decreased by 1.98 ± 0.45-fold as a result of treatment with clofibrate (Fig. 3.4.1, panels A, B and C, compare lanes 1 and 2 to lanes 3 and 4).

The varied expression of the peroxisomal membrane proteins upon clofibrate treatment parallels that of the matrix enzymes. While the matrix enzymes involved in fatty acid β-oxidation are significantly induced, the other known peroxisomal matrix enzymes are affected differently. Catalase activity undergoes a slight increase (1.26-fold, Hess *et al*, 1965; and 1.53-fold, Lazarow and de Duve, 1976), while D-amino acid oxidase specific activity decreases 5.75-fold (Hayashi *et al*, 1975) and urate oxidase activity decreases by 2.36-fold (Hess *et al*, 1965) upon treatment with clofibrate. It was suggested that decreases in specific activity actually reflect dilution due to the increase in liver weight (Hess *et al*, 1965) rather than down regulation of gene expression. This is

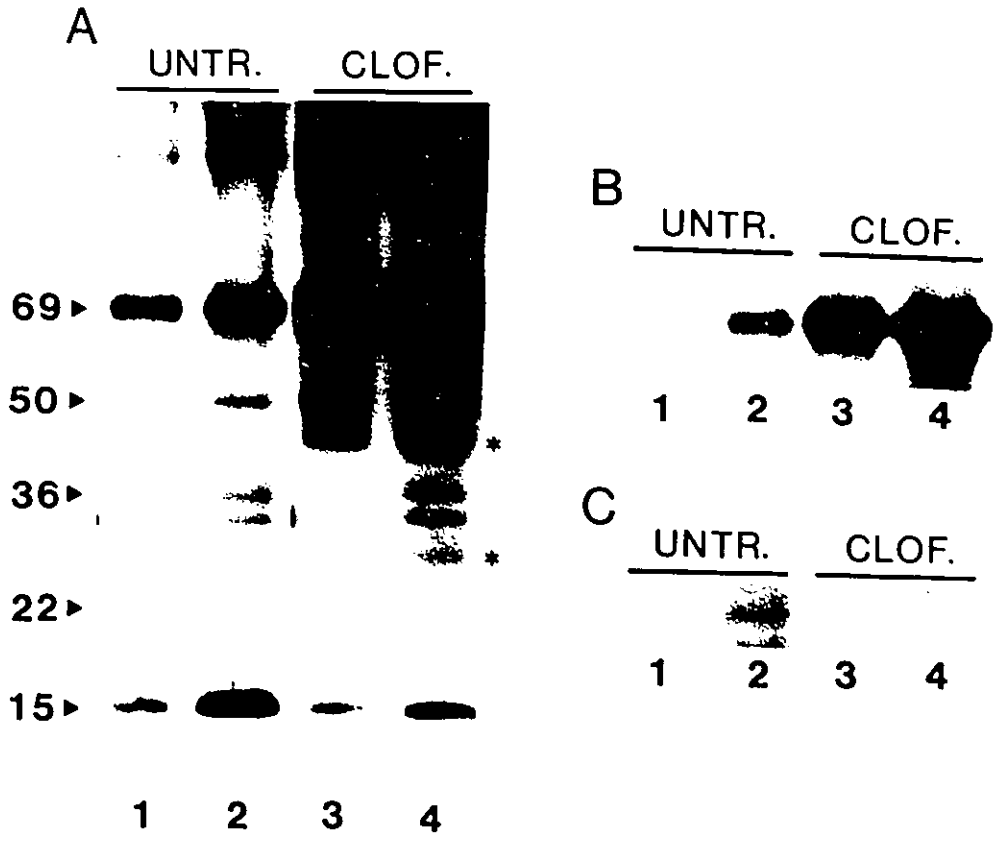



Fig. 3.4.1. Immunoblot analysis of rat liver peroxisomal membranes from untreated (lanes 1 and 2) and clofibrate-treated rats (lanes 3 and 4) with anti-peroxisomal IMP serum. Peroxisomal IMPs (20 μg in lanes 1 and 3 and 40 μg in lanes 2 and 4) were separated by SDS-PAGE prior to transfer. Molecular masses of the IMPs of interest are indicated at the left (in kDa). Panel A was exposed to Kodak X-OMAT AR film for 60 hours. Panel B shows the 69 kDa IMP exposed for 24 hours and panel C shows the 22 kDa IMP exposed for 120 hours. The bands marked with the asterisks are thought to be the result of proteolysis of the 69 kDa IMP.

supported by evidence that the mRNA encoding urate oxidase is increased 2- to 3- fold upon clofibrate treatment (Reddy *et al*, 1988), even though the specific activity of the enzyme decreases. Concomitant increases in amounts of the 69 kDa IMP and the peroxisomal ß-oxidation enzymes implicates a role for the 69 kDa IMP in peroxisomal lipid metabolism. It has been postulated that the 69 kDa IMP may function in the translocation of acyl-CoA compounds across the peroxisomal membrane, as acyl-CoA synthetase activity has been localized to the cytoplasmic face of the peroxisomal membrane (Hartl and Just, 1987). However, direct evidence for the function of the 69 kDa IMP is lacking. It remains to be determined if the increase in 69 kDa IMP is the result of an increase in transcription of its gene, as is the case for the peroxisomal ß-oxidation enzymes (Reddy *et al*, 1986).

## 3.5 Intracellular Site of Synthesis of the Peroxisomal IMPs

The intracellular site of synthesis of the IMPs was determined by immunoprecipitation of *in vitro* translation products of RNA isolated from free and membrane-bound rat liver polysomes. Free and membrane-bound polysomes were isolated from the livers of clofibrate-treated rats by the procedure of Ramsey and Steele (1976) as modified by Rachubinski *et al* (1980). RNA was isolated from the polysomes by the guanidine thiocyanate-CsCl procedure described by

Raymond and Shore (1979). The polypeptide profiles of the translation products of the free and membrane-bound RNA fractions were very distinct, shown in Fig. 3.5.1. The purity of the fractions was estimated by the proportion of rat serum albumin, a secreted protein, synthesized in the membrane-bound polysome fraction. Rat serum preproalbumin was readily recognized at a molecular weight of 68,000, because its synthesis constitutes 2-12% of hepatic protein synthesis (McLaughlin and Pitot, 1976). By densitometric analysis, 88% of rat serum preproalbumin was made on membrane-bound polysomes.

Immunoselection of *in vitro* translation products of free and membrane-bound RNA with the anti-peroxisomal IMP serum illustrated that the 69 kDa, 36 kDa and the 22 kDa IMPs were preferentially synthesized on free polysomes (Fig. 3.5.2, compare lanes 2 and 3). Each of these was synthesized without a detectable precursor extension, as the *in vitro* synthesized polypeptides co-migrated with the mature IMPs isolated from peroxisomes. Interestingly, this experiment indicated that the 50 kDa IMP was preferentially synthesized on membrane-bound polysomes (Fig. 3.5.2, compare lanes 2 and 3, indicated by the solid arrowhead). Densitometric analysis showed that 81% of the 50 kDa polypeptide was synthesized on membrane-bound polysomes. As a control, 92% of urate oxidase, the 33 kDa peroxisomal protein, was synthesized on free polysomes (Goldman and Blobel, 1978; Robbi and Lazarow, 1978) (Fig.

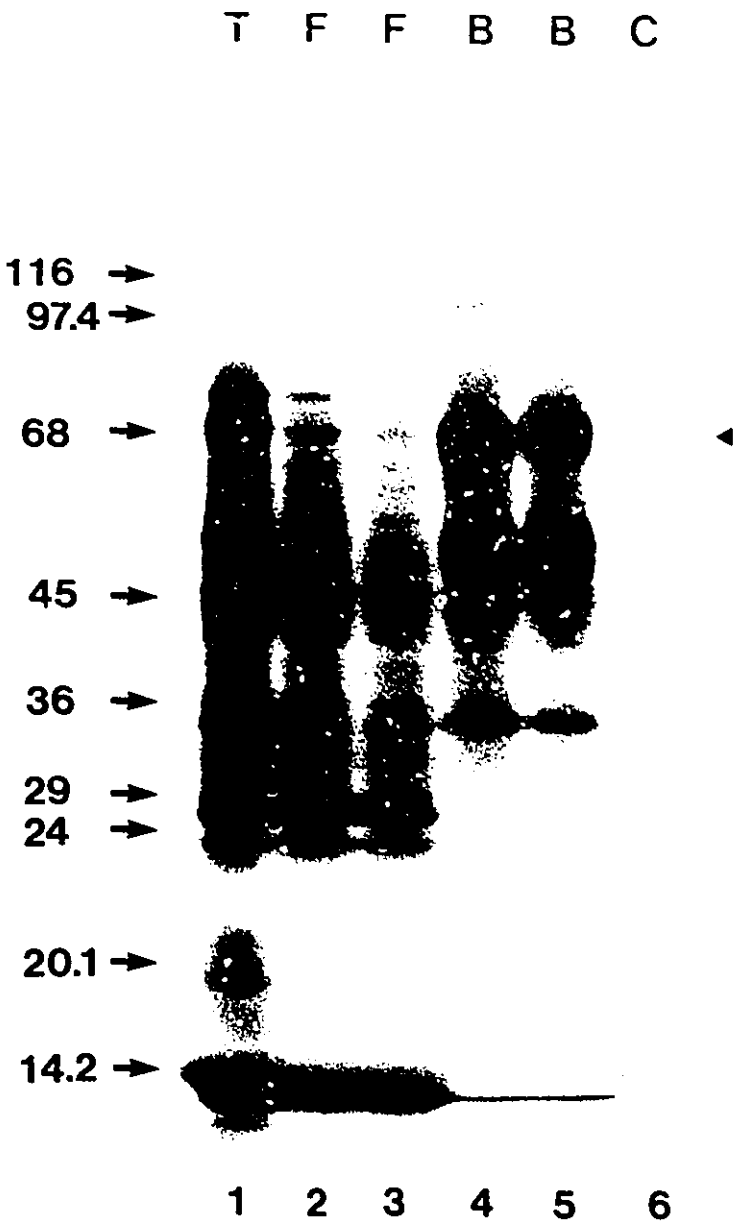



Fig. 3.5.1. Polypeptide profiles of the translation products of total, free and membrane-bound RNA fractions. Fluorogram of cell-free translation products from total RNA (T, lane 1), free polysomal RNA (F, lanes 2 and 3), membrane-bound polysomal RNA (B, lanes 4 and 5) and control translation (no RNA added, C, lane 6). Molecular mass standards are indicated at the left. The arrowhead at the right indicates the migration of rat serum preproalbumin.

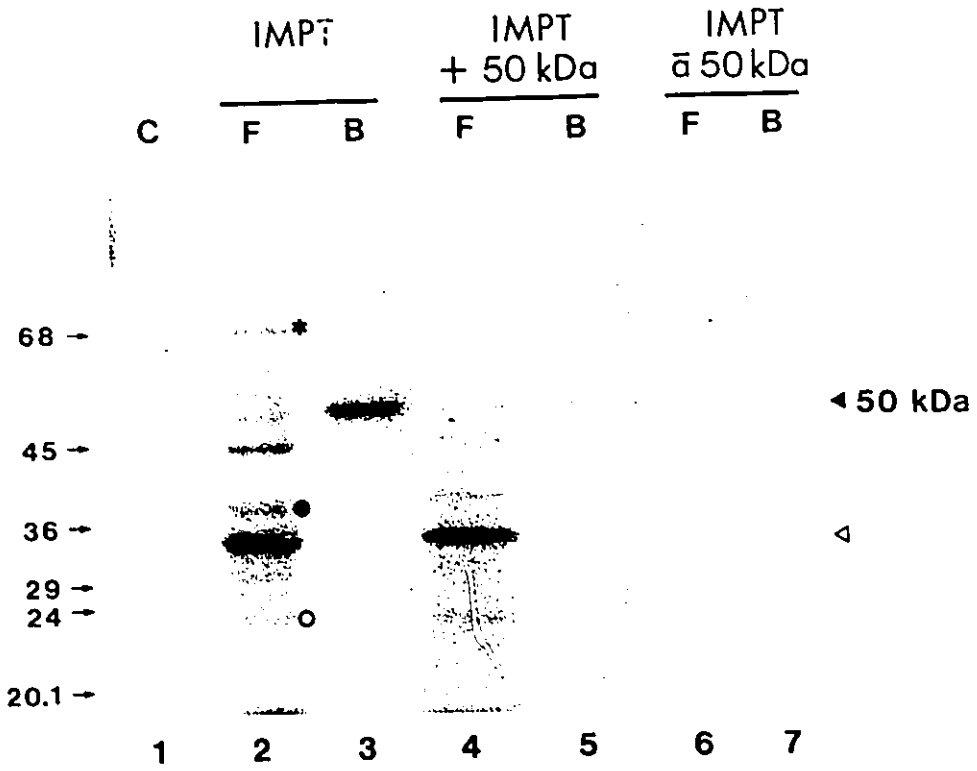



Fig. 3.5.2. Intracellular sites of synthesis of the peroxisomal IMPs. Fluorogram of cell-free translation products of free (F) polysomal RNA (lanes 2 and 4) and membrane-bound (B) polysomal RNA (lanes 3 and 5) immunoselected with anti-peroxisomal IMP serum. Competition with unlabeled 50 kDa IMP (20 μg) during immunoprecipitation (lanes 4 and 5). Immunoselection of cell-free translation products of free (lane 6) and membrane-bound polysomal RNA (lane 7) with monospecific antibodies to the 50 kDa IMP. Control immunoprecipitation of total RNA translation products with pre-immune serum (lane 1). The solid arrowhead (◂) indicates the 50 kDa IMP. The open arrowhead (◁) indicates urate oxidase. The asterisk (*) indicates the 69 kDa IMP. The solid circle (●) is the putative 36 kDa IMP and the open circle (o) indicates the 22 kDa IMP.

3.5.2, compare lanes 2 and 3, indicated by the open arrowhead). The 50 kDa IMP was apparently synthesized with either a small or no precursor extension, as the *in vitro* synthesized polypeptide co-migrated with the mature percxisomal 50 kDa polypeptide in SDS-PAGE. To confirm the identity of the immunoprecipitated *in vitro* translation product as the 50 kDa peroxisomal IMP, unlabeled 50 kDa IMP (electroeluted from SDS-polyacrylamide gels) was added in excess during the immunoprecipitation. The radiolabeled 50 kDa polypeptide was specifically eliminated by competition (greater than 90% by densitometric analysis) by the unlabeled 50 kDa IMP (Fig. 3.5.2, compare lanes 2 to 4 and 3 to 5). Immunoprecipitation of free and membrane-bound RNA translation products with affinity-purified, monospecific 50 kDa antiserum (Fig. 3.5.2, lanes 6 and 7) further verified that the 50 kDa polypeptide was synthesized on membrane-bound polysomes.

The site of synthesis of the 15 kDa IMP could not be determined in these experiments due to background bands in this area of the gel. The identities of the 69 kDa, 36 kDa and 22 kDa IMPs were not confirmed by competition experiments; however, it has since been reported that the 69 kDa and 22 kDa IMPs are preferentially synthesized on free polysomes without precursor extensions (Suzuki *et al*, 1987; Hartl and Just 1987). There is some question as to the identity of the 36 kDa IMP, because we cannot rule out the possibility that the polypeptide at this molecular weight is a fragment derived

from the 69 kDa polypeptide. The additional polypeptides which were immunoprecipitated from the translation products of free polysomal RNA (molecular masses 45 kDa and 30 kDa) may have resulted from proteolysis of the 69 kDa IMP. These two polypeptide bands have appeared in previous experiments (Fig. 3.4.1, panel A, lanes 3 and 4), and Hartl and Just (1987) have reported that a 42 kDa IMP and a 28 kDa IMP are derived from the 69 kDa IMP.

The most widely accepted model of peroxisome biogenesis proposes that all peroxisomal proteins are synthesized on free polysomes and posttranslationally imported and that new peroxisomes are formed by fission of existing peroxisomes (Lazarow and Fujiki, 1985). From investigations of the peroxisomal IMPs, it appeared that the 69 kDa, 36 kDa and 22 kDa IMPs follow this model; however, the 50 kDa IMP is enigmatic. The 50 kDa IMP was co-localized to peroxisomes and ER. Immunoprecipitation of *in vitro* translation products of RNA isolated from free polysomes and membrane-bound polysomes indicated that the 50 kDa IMP was preferentially synthesized on membrane-bound polysomes. Proteins that are targeted to both peroxisomes and ER present a unique problem concerning their site of synthesis in the cell and mode of transport to these distinct organelles. In the case of the 50 kDa IMP there are a few explanations for the observed results. There may be two isoforms of the 50 kDa IMP that share common epitopes but have structural differences which result in one

isoform being targeted to the ER and the other to the peroxisome. In this case the ER isoform may be synthesized on membrane-bound polysomes, while the other isoform may be synthesized on free polysomes and subsequently targeted to the peroxisome. Contamination of the free polysomal RNA fraction with membrane-bound polysomal RNA was approximately 10%. When this is taken into account it suggests that approximately 9% of 50 kDa may be synthesized on free polysomes. This fraction may be targeted to peroxisomes, while the other approximately 91% of 50 kDa, synthesized on membrane-bound polysomes, may be targeted to the ER. Alternatively, the possibility exists that there is only one 50 kDa IMP which is synthesized on membrane-bound polysomes, and there exists a route of transfer of that protein from the ER to peroxisomes. This would suggest a role for the ER in peroxisome biogenesis, but at this time we cannot rule out the possibility that the large fraction of the 50 kDa IMP synthesized on membrane-bound polysomes is targeted exclusively to the ER and that the small fraction synthesized on free polysomes is targeted to peroxisomes. The composition of normal rat hepatocytes consists of 21.5% ER protein and only 2.53% peroxisomal protein, which may explain the large proportion of 50 kDa synthesized on membrane-bound polysomes. However, the 50 kDa IMP appears to be more abundant in the peroxisomal membrane, as determined by signal intensity on immunoblots and the fact that predominantly peroxisomes are detected in

immunofluorescence microscopy analysis with monospecific antibodies to the 50 kDa IMP. The difference in amounts of 50 kDa in the ER membrane as compared to the peroxisomal membrane may be due to rapid turnover of this protein in the ER or exit of this protein to another compartment.

The 36 kDa IMP which was co-localized to peroxisomal and ER membranes was tentatively identified as being synthesized on free polysomes. This protein may have two isoforms, one containing a peroxisomal targeting signal and the other containing ER targeting potential. However, the ER isoform does not use the traditional machinery for proteins entering the ER. Examples of proteins targeted to the secretory system which do not use the traditional ER import machinery are the RAS-like GTP-binding proteins and the mating pheromones of some yeasts and fungi (Powers *et al*, 1986; Pugsley, 1989). Alternatively, it is possible that there is only one 36 kDa protein synthesized on free polysomes that harbours targeting information for both peroxisomes and ER.

Although the site of synthesis of the 15 kDa IMP was not determined, its distribution in the membranes of all subcellular fractions investigated (peroxisomes, mitochondria, lysosomes and ER) suggests that it possesses a more non-specific targeting potential, perhaps similar to that of cxytochrome $b_5$ (Rachubinski *et al*, 1980). Cytochrome $b_5$ is an integral membrane protein with ubiquitous distribution to intracellular membranes (ER, Golgi, plasmalemma, mitochondria,

peroxisomes and nuclear envelope), which has been shown to be synthesized on free polysomes. Following its release from the ribosome it inserts non-selectively into nearby membranes by virtue of its C-terminal, hydrophobic membrane-insertion sequence (Dailey and Strittmatter, 1978; Fleming and Strittmatter, 1978).

In addition to the 15 kDa, 36 kDa and 50 kDa IMPs, other enzymes have been identified which are localized to peroxisomes and other subcellular compartments. Some of these enzymes and their intracellular locations are given in Table 3.5.1. In general, proteins that are targeted to more than one subcellular compartment raise some important questions in cell biology:

1) How are these proteins targeted to distinct subcellular destinations?

2) Why are similar enzymes located in more than one site in the cell?

With regard to the first question, the possibility exists that there is more than one gene, each encoding different isoforms of the protein with different targeting potential. Alternatively, there may only be one gene, and differential transcriptional or post-translational processes may generate different forms of the protein. This is the case for rat liver peroxisomal and mitochondrial serine:pyruvate aminotransferase (SPT). Peroxisomal and mitochondrial SPT share identical physical properties but differ in their

Table 3.5.1. Rat Liver Enzymes Localized to Peroxisomes and Other Subcellular Organelles.

| Enzyme | Subcellular Locations | Reference |
|---|---|---|
| carnitine acetyltransferase | peroxisomes, mitochondria, ER | Markwell et al, 1977 |
| carnitine octanoyltransferase | peroxisomes, mitochondria, ER | Markwell et al, 1977 |
| long-chain acyl-CoA synthetase (palmitoyl CoA ligase) | peroxisomes, mitochondria, ER | Miyazawa et al, 1985 |
| very long-chain acyl-CoA syntnetase (lignoceroyl-CoA ligase) | peroxisomes, ER | Singh et al, 1987 |
| sterol carrier protein 2 (nonspecific lipid transfer protein) | peroxisomes, mitochondria, ER, cytosol | Keller et al, 1989 |
| 3-hydroxy-3-methylglutaryl-CoA reductase | peroxisomes, ER | Keller et al, 1985 |
| bile acid-CoA: amino acid N-acyltransferase | peroxisomes, ER | Kase and Björkhem, 1989 |
| acyl dihydroxyacetone-phosphate: NADPH oxidoreductase | peroxisomes, ER | Hajra et al, 1979 |
| NADPH cytochrome P-450 reductase | peroxisomes, ER | Gutierrez et al, 1988 |
| NADH cytochrome c($b_5$) reductase | peroxisomes, mitochondria, ER, Golgi | Gutierrez et al, 1988; Borgese and Gaetani (1980) |
| cytochrome $b_5$ | peroxisomes, mitochondria, ER, Golgi, plasmalemma, nuclear envelope | Rachubinski et al, 1980 |
| AlaATgl (aminotransferases including: serine:pyruvate aminotransferase phenylalanine:pyruvate aminotransferase histidine:pyruvate aminotransferase) | peroxisomes, mitochondria | Noguchi and Fujiwara 1988 |

subcellular locations. A study by Oda *et al* (1990) revealed that two different mRNAs are generated from a single gene through initiation of transcription from different start sites. The longer mRNA encodes the precursor for mitochondrial SPT, which has an amino-terminal extension of 22 amino acids containing a mitochondrial targeting signal. The amino-terminal extension is processed in the mitochondrion to generate a protein identical to the peroxisomal SPT. Presumably, the shorter gene product is translocated into peroxisomes by a peroxisomal targeting signal located in the protein which is recognized in the absence of the mitochondrial signal (Oda *et al*, 1990). One can envision other processes such as differential splicing of a transcript or differential posttranslational processing of a polypeptide which could generate different forms of a protein each harbouring distinct targeting information. In addition to these types of mechanisms, a more non-specific mechanism of targeting may exist as in the case of cytochrome $b_5$.

With regard to the second question, it is interesting that there is an overlap of function of many metabolic pathways or certain steps of metabolic processes among the various cellular compartments. For example, ß-oxidation of fatty acids occurs in both peroxisomes and mitochondria (Lazarow and de Duve, 1976). In addition, some processes in cholesterol biosynthesis and bile acid synthesis occur in the ER and peroxisomes (Appelkvist *et al*, 1990; Kase *et al*, 1983).

The ß-oxidation pathways and the enzymes involved in these processes are well characterized. Although these enzymes perform similar functions, they have different molecular and catalytic properties. The existence of the two pathways may provide maximum efficiency of ß-oxidation of different substrates. For example, the ß-oxidation of long chain or unsaturated fatty acids may occur preferentially in peroxisomes (Osmundsen, 1982). In addition, similar pathways in different locations may allow the cell to partition substances to distinct metabolic fates. For example, while the fatty acid ß-oxidation pathway in mitochondria provides acetyl-CoA for subsequent energy production, it has been suggested that the peroxisomal ß-oxidation pathway provides acetyl-CoA units as substrates for cholesterol biosynthesis, bile acid synthesis or phospholipid synthesis (Hayashi and Takahata, 1991). Alternatively, multiple discrete pathways may provide additional cellular regulation at more than one site, as has been suggested for cholesterol biosynthesis (Keller *et al*, 1985). In this case, there must be good coordination of the functions between the various organelles, but how this is achieved is unknown.

Recent studies have described close physical contacts between different organelles, and these associations have been implicated in the coordination of functions between organelles. In particular, some studies have described close membrane associations between peroxisomes and the ER (Zaar *et*

*al*, 1987; Baumgart *et al*, 1989). Zaar *et al* (1987) showed close membrane contacts between the ER and peroxisomes in bovine kidney cortex epithelial cells by cytochemical staining for glucose-6-phosphatase in peroxisome-associated membrane structures. Subsequently, Baumgart *et al* (1989) observed close membrane associations between the ER and peroxisomes by electron microscopy. Zaar *et al* (1987) suggest that the close membrane associations between ER and peroxisomes may facilitate a functional cooperation between these two compartments. They further propose a shuttle of lipid intermediates between ER and peroxisomes may exist in the synthesis of complex lipids. For example, peroxisomes contain some of the major enzymes involved in the synthesis of ether-linked glycerolipids. However, enzymes catalyzing the terminal reactions in the synthesis of ether-linked glycerolipids are found in microsomes (Hajra and Bishop, 1982; Zaar *et al*, 1987). Baumgart *et al* (1989) postulate that the peroxisome-ER contacts may represent areas which facilitate the transfer of newly synthesized phospholipids from the sER to specialized membranous structures that are continuous with peroxisomes. At present, because all peroxisomal proteins studied are synthesized on free polysomes and posttranslationally inserted into peroxisomes, an involvement of peroxisome-ER complexes in the synthesis and transport of peroxisomal proteins has not been shown (Zaar *et al*, 1987). However, the discovery of an immunoreactive IMP localized to

peroxisomes and ER and synthesized predominantly on membrane-bound polysomes raises interesting possibilities concerning the role of the ER in peroxisome biogenesis. However, the exact mechanism by which the 50 kDa IMP gains residence in the peroxisomal membrane remains to be elucidated.

## 3.6 Summary and Future Directions

In summary, the data presented help to characterize further the IMPs of rat liver peroxisomes. The 69 kDa IMP was significantly increased upon clofibrate treatment, whereas the other IMPs remained unchanged (22 kDa and 36 kDa), increased (50 kDa) or decreased (15 kDa) to a lesser extent. This result implicates a role for the 69 kDa IMP in the ß-oxidation pathway. The 69 kDa and 22 kDa IMPs were localized exclusively in the peroxisomal membrane. The 15 kDa IMP was found in the membranes of peroxisomes, lysosomes, mitochondria and ER, and the 36 kDa and 50 kDa IMPs were co-localized to the peroxisome and ER. The multiorganellar location of these IMPs is not surprizing, as many proteins have been co-localized to peroxisomes and other organelles (Table 3.5.1). However, the significance of this result with respect to peroxisome function or biogenesis remains to be determined. Are these immunologically related IMPs the same protein or isoforms of a protein, and how are they targeted to these distinct organelles? The 22 kDa, 36 kDa and 69 kDa IMPs appeared to be synthesized predominantly on free polysomes;

however, the 50 kDa IMP was preferentially synthesized on membrane-bound polysomes.

These observations raise a number of questions that could be addressed by future experimentation. It would be of interest to learn about the targeting signals within the peroxisomal IMPs that are responsible for directing these proteins to peroxisomes. This would involve cloning the cDNAs encoding these proteins and using a mutational approach to determine which segments of the protein are essential for import, using either a heterologous *in vivo* approach (see Results and Discussion, section 4.7) or an *in vitro* import assay. In addition, it would be of interest to characterize the cytosolic or membrane components that facilitate targeting to the membrane. Previous studies have indicated that the targeting pathway of peroxisomal IMPs may differ from that of the matrix proteins. It has been shown that cells of patients with Zellweger syndrome contain "peroxisomal ghosts" consisting of peroxisomal membranes that contain the integral membrane proteins but which are unable to import the matrix proteins (Santos *et al*, 1988a; 1988b). Understanding peroxisomal biogenesis will involve elucidating the details of the targeting signals and other components involved in each import pathway.

With regard to the proteins having multiorganellar locations, it would be of interest to determine if they are identical proteins or different isoforms. This would involve

isolating the proteins from their respective subcellular locations and characterizing their properties. In addition, studying the genes encoding these proteins would lend understanding as to how the proteins arise:

1) two genes encoding two different proteins with different targeting potential

2) one gene giving rise to two transcripts

3) one gene encoding a protein with targeting information for more than one subcellular location.

Cloning the cDNAs encoding these proteins could help address these questions.

In the case of the 50 kDa IMP, pulse-chase studies would allow one to follow its biosynthesis from the site of synthesis to its final destination in the cell in order to determine if it first passes through the ER *en route* to peroxisomes.

It would be important to elucidate the functions of these IMPs. Hints about a protein's function can be implied from studying its properties; however, the ultimate test will be the use of reconstitution experiments or classical genetic approaches by which one can correlate loss of function with a particular mutation.

In order to facilitate many of these future experiments, the cloning of some of the cDNAs encoding the rat liver peroxisomal IMPs was undertaken. Partial cDNA recombinants potentially encoding the 50 kDa IMP and the 69

kDa IMP were isolated by immunoselection from a λgt11 rat liver expression library using monospecific, affinity-purified antibodies to these proteins. Although the results are somewhat preliminary, the characterization of the partial cDNA recombinant encoding the 50 kDa IMP is presented in Appendix A.

4. Cloning and Sequence Determination of a cDNA Encoding a Second Rat Liver Peroxisomal 3-Ketoacyl-CoA Thiolase

3-ketoacyl-CoA thiolase (thiolase) catalyzes the final step of the fatty acid ß-oxidation pathway in peroxisomes. Thiolase is an exception among rat liver peroxisomal enzymes in that it is synthesized as a precursor possessing an amino-terminal extension which is cleaved to generate the mature enzyme (Fujiki et al, 1985; Hijikata et al, 1987). It is not clear whether the proteolytic processing of thiolase is functionally coupled to import into peroxisomes. In Zellweger syndrome fibroblasts (a condition characterized by defective peroxisomal protein import), the thiolase precursor is formed and is located in the cytosol but is not cleaved to the mature form before being degraded (Schram et al, 1986). This result suggests that cleavage of the amino-terminal extension of thiolase is dependent on a functional peroxisomal protein import system. In contrast, pulse-labeling and subcellular fractionation experiments with rat hepatocytes revealed that the precursor and mature forms of thiolase are present in both cytosolic and particulate fractions (Miura et al, 1984). This suggests that the processing of thiolase is not linked to import (Miura et al, 1984).

To further investigate the synthesis, intracellular transport and processing of this enzyme, the molecular cloning

of the cDNA encoding peroxisomal thiolase was undertaken. Upon sequencing several cDNA recombinants for thiolase, it was discovered that there are two similar, but distinct thiolase enzymes in rat liver peroxisomes. Interestingly, the expression of the genes encoding the two thiolases was differentially regulated. One appeared to be constitutively expressed in rat liver and was only slightly induced by treatment of rats with clofibrate (thiolase 2). The other (thiolase 1) was expressed at a very low level in normal rat liver and was highly induced upon treatment with clofibrate.

The work presented in this section facilitated *in vivo* targeting studies to determine the role of the thiolase presequence in directing this protein to peroxisomes (section 4.7).

## 4.1 Preparation and Characterization of Anti-Thiolase Serum

Thiolase, a peroxisomal matrix protein ($M_r$ 41 000), was isolated from purified peroxisomes by preparative SDS-PAGE and used to raise an antiserum in rabbits. Immunoblot analysis indicated that the anti-thiolase serum reacted exclusively with peroxisomal thiolase and did not cross-react with mitochondrial thiolase (Fig. 4.1.1). This is in agreement with previous data that show that peroxisomal 3-ketoacyl-CoA thiolase, mitochondrial 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase are immunologically unrelated

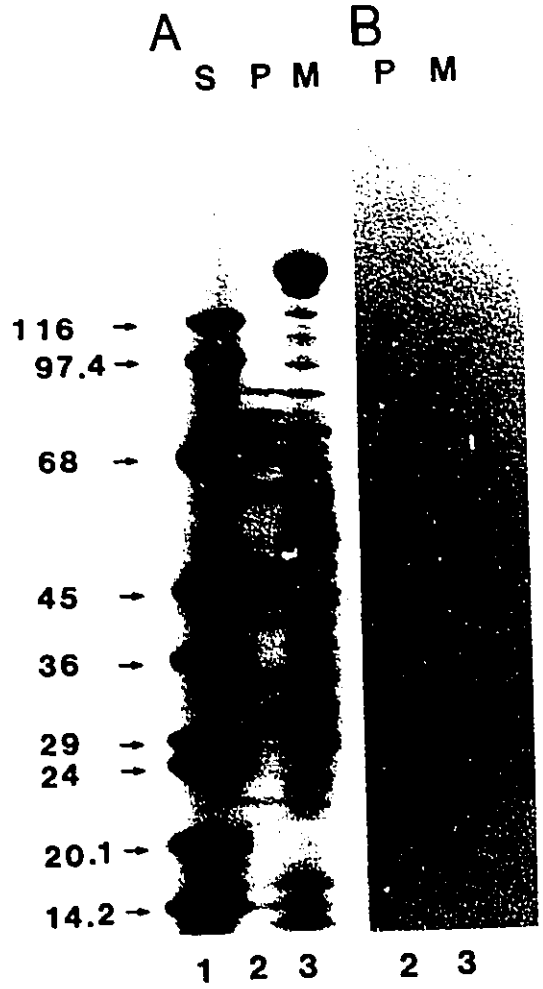



Fig. 4.1.1. Immunoblot analysis of rat liver peroxisomes and mitochondria with anti-peroxisomal thiolase serum. Panel A, SDS-polyacrylamide gel stained with Coomassie Brilliant Blue; panel B, corresponding immunoblot. Lane 1, proteins standards (S), molecular masses (in kDa) are indicated at the left; lane 2, peroxisomes (P) (20 μg); lane 3, mitochondria (M) (20 μg).

(Miyazawa *et al*, 1980). Subsequent immunoblots, using peroxisomes isolated from untreated and clofibrate-treated rats, revealed that not only was thiolase induced upon treatment with clofibrate (Fig. 4.1.2, compare lanes UP and CP) but that the anti-thiolase serum reacted with a second distinct protein band of similar yet resolvable size (Fig. 4.1.2, panel B, lane CP, indicated by the arrowheads). This second protein band was only discernible when the peroxisomal fraction was electrophoresed on extended SDS-polyacrylamide gels (14 cm x 28 cm, instead of the usual 14 cm x 12 cm). The origin of the second protein band was not conclusively determined. The lower protein band could represent another form of thiolase or perhaps be the result of proteolysis of mature thiolase.

## 4.2 Selection and Sequencing of cDNA Recombinants Encoding Rat Liver Peroxisomal Thiolase

The anti-thiolase serum was used to select cDNA recombinants from a λgt11 rat liver expression library by the double-antibody technique of Young and Davis (1983). Immunoscreening yielded three positive recombinants (λT3, λT4 and λT6) from the approximately 10,000 plaques screened (Fig. 4.2.1). Identification of a positive recombinant depends upon creating an in-frame fusion with the ß-galactosidase structural gene, *LacZ*. Considering there is a one in six chance of obtaining the correct reading frame, we can estimate

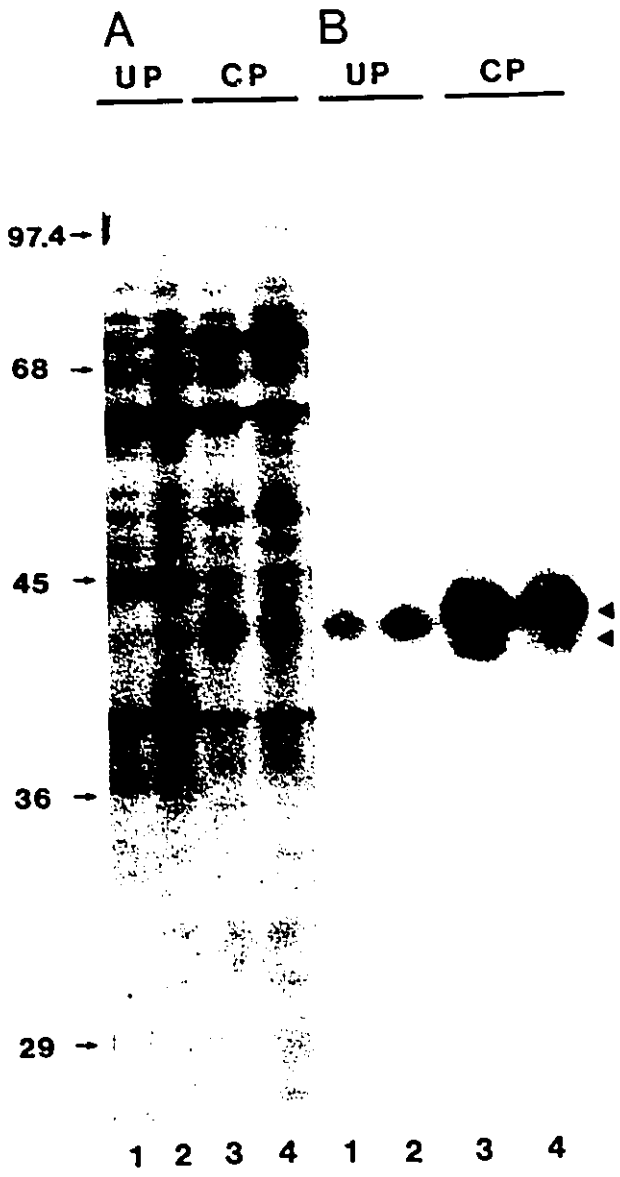



Fig. 4.1.2. Immunoblot analysis of peroxisomes isolated from untreated and clofibrate-treated rat liver with anti-thiolase serum. Panel A, SDS-polyacrylamide gel stained with Coomassie Brilliant Blue; Panel B, corresponding immunoblot. Lane 1 and 2, peroxisomes isolated from untreated rats (UP) (30 μg); lane 3 and 4, peroxisomes isolated from clofibrate-treated rats (CP) (30 μg). Molecular mass protein standards (in kDa) are indicated at the left. Arrowheads at the right indicate the two protein bands that react with anti-thiolase serum.

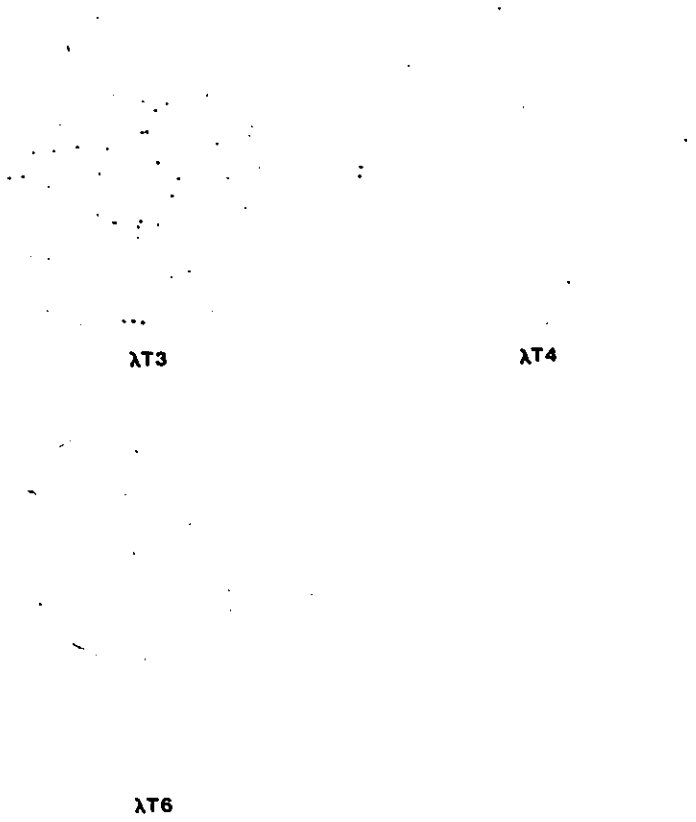



Fig. 4.2.1. Immunoscreening of the λgt11 rat liver cDNA library with anti-thiolase serum. Nitrocellulose discs representing the secondary screens enriched for the positive recombinants λT3, λT4 and λT6, respectively, were treated with a 1:1000 dilution of rabbit anti-rat peroxisomal thiolase serum, then with a 1:7500 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate and finally with the substrates BCIP and NBT to produce dark purple plaques in a positive reaction.

the abundance of peroxisomal thiolase mRNA to be approximately 0.18% in normal rat liver. Digestion of the phage DNA with the restriction endonuclease *Eco*RI revealed that recombinant λT3 had a 0.8 kbp cDNA insert, recombinant λT4 had two fragments of 0.4 and 1.1 kbp and recombinant λT6 had two fragments of 0.6 and 1.0 kbp. These recombinants were partially digested with *Eco*RI to liberate a proportion of cDNA inserts without cutting the internal *Eco*RI sites (recombinants λT4 and λT6) and the digestion mixture was subcloned into the *Eco*RI site of pGEM-7Zf(+) (Fig. 4.2.2). Recombinant plasmids were selected which contained the entire cDNA inserts (0.8, 1.5, 1.6 kbp) and designated pT3, pT4-4 and pT6, respectively. These cDNA inserts were sequenced from both strands of DNA by the nested deletion strategy of Henikoff (1984). The first set of nested deletion fragments was constucted by digesting the plasmids in the multiple cloning site with *Xho*I and *Sph*I. This created an exonuclease III (exo III) sensitive 5'-overhang (*Xho*I site) adjacent to the insert and an exo III resistant 3'-overhang (*Sph*I site) adjacent to the M13 universal primer binding site. The deletions were created by unidirectional digestions with exo III, polishing the DNA ends with S1 nuclease and Klenow fragment and ligating the blunt ends back together. The second set of nested deletions was created in a similar manner but the initial digestions were performed with *Sac*I and *Cla*I located at the other side of the cDNA insert (Fig 4.2.2). The cDNA recombinants were sequenced

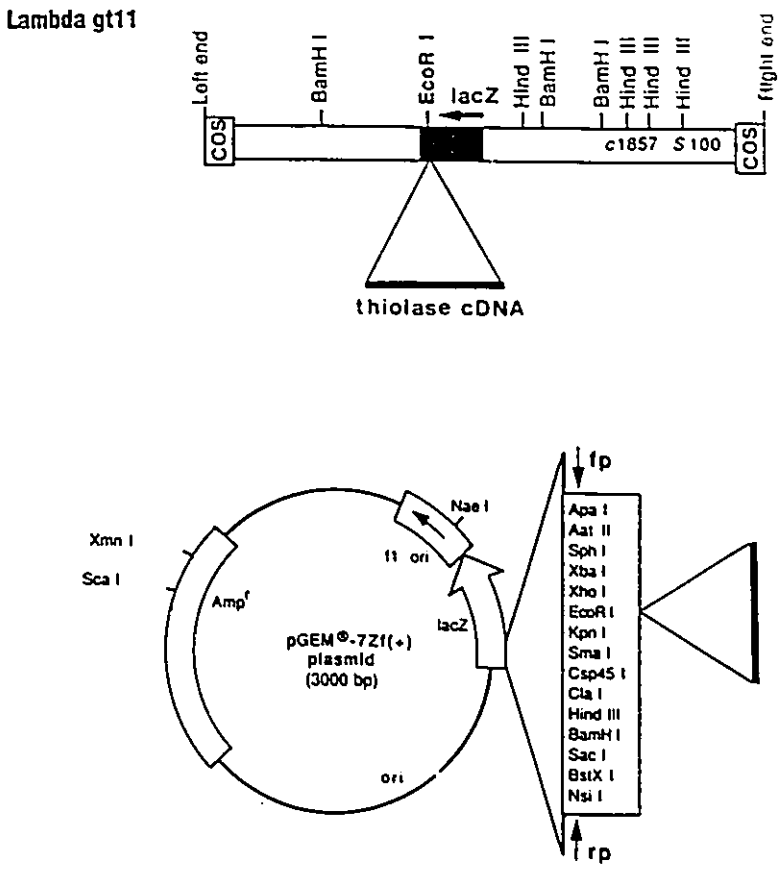



Fig. 4.2.2. Schematic representation of the λgt11 and pGEM7Zf(+) vectors employed in cloning rat liver peroxisomal thiolase cDNA. A partial restriction map of λgt11 is illustrated at the top. The *lacZ* gene is darkened and the direction of transcription is indicated by the arrow. The unique *Eco*RI site is indicated as well as the temperature-sensitive cI repressor (c1857), the amber mutation (S100) and the cohesive ends (COS). A partial restriction map of pGEM-7Zf(+) is shown at the bottom. The *lacZ* gene and its direction of transcription, the region coding for β-lactamase ($Amp^r$), the phage f1 region (f1 ori) and bacterial ori are indicated. The restriction endonuclease sites present in the multiple cloning site appear in the boxed region. The binding site of the pUC/M13 forward sequencing primer (fp) and the pUC/M13 reverse sequencing primer (rp) are shown. The thiolase cDNA is represented by the thick line and the site of insertion into each vector is indicated. These figures were adapted from the Promega catalogue.

using the double-stranded dideoxy chain-termination procedure of Zhang et al, 1988. Sequencing the partial cDNAs identified two distinct sets of recombinants. The sequence of recombinant pT6 was identical to that of the previously published rat liver peroxisomal thiolase (thiolase 1) (Hijikata et al, 1987); however, pT4-4 and pT3 possessed a similar yet distinct nucleotide sequence. Because the cDNA recombinants pT4-4 and pT3 were selected with the antibody which reacted exclusively with peroxisomal thiolase and shared a high level of homology with the previously published cDNA sequence encoding rat liver peroxisomal thiolase, we proposed that these cDNA recombinants correspond to a newly identified rat liver peroxisomal thiolase, referred to as thiolase 2. Subsequent work concentrated on completing and characterizing this novel thiolase 2 cDNA. The largest thiolase 2 cDNA recombinant (pT4-4) was found to be 1435 bp in length, containing a single open reading frame of 1194 nucleotides and lacking the 5'-end of the cDNA. A partial restriction map and a sequencing strategy for recombinant pT4-4 are shown in Fig. 4.2.3.

### 4.2.1 Primer Extension of the Thiolase 2 mRNA

Primer extension of the mRNA encoding thiolase 2 was used to complete the 5'-end of the thiolase 2 cDNA. The primer extension strategy termed "rapid amplification of cDNA ends" (RACE, Fig. 4.2.4) was employed (Frohman et al, 1988).

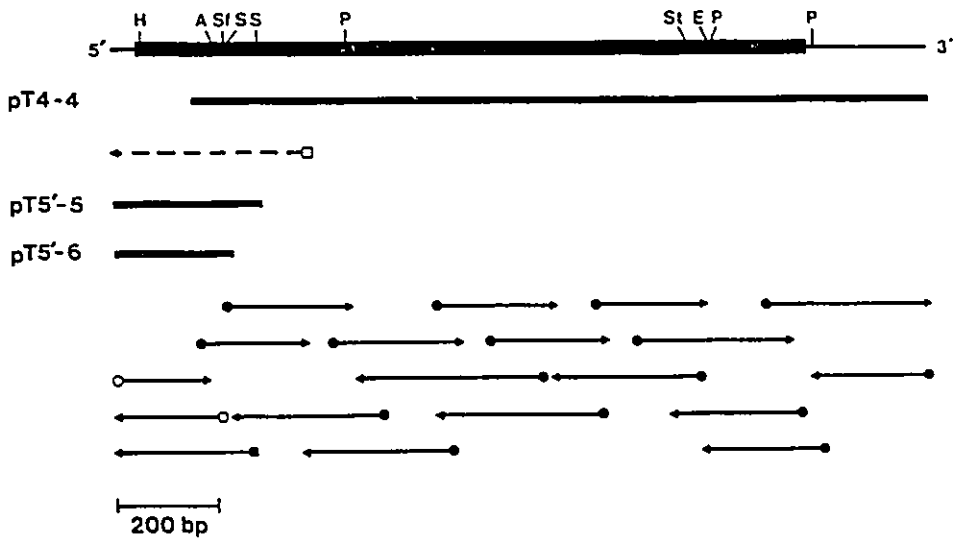



Fig. 4.2.3. Partial restriction map and sequencing strategy for the cDNA encoding rat liver peroxisomal thiolase 2. The composite cDNA is shown at the top with the open reading frame indicated by a thick line. A, *Apa*LI; E, *Eco*RI; H, *Hinf*I; P, *Pst*I; S, *Sst*II; Sf, *Sfi*I; St, *Stu*I. pT4-4 was the largest partial recombinant encoding thiolase 2 immunoselected from the λgt11 rat liver library. It was constructed by subcloning the cDNA insert into the vector pGEM-7Zf(+). The oligo used to direct RACE primer-extension is shown by an open box. The direction and extent of primer-extended cDNA are indicated by the dashed arrow. The resultant cDNA products were amplified by PCR, digested with *Sst*II and *Sal*I (the *Sal*I site is present in the adaptor at the 5′-end of the cDNA) and subcloned into the pGEM-5Zf(+) vector. Plasmids pT5′-5 and pT5′-6 contain cDNA inserts from two indepenent PCR reactions. Direction and extent of sequence determination are indicated by arrows. The solid circles represent the starting points of dideoxy chain-termination sequencing of nested deletion fragments that were created with exonuclease III using recombinant pT4-4. The arrows with open circles represent sequence derived from recombinant pT5′-6. The arrow with a solid square indicates the sequence from recombinant pT5′-5.

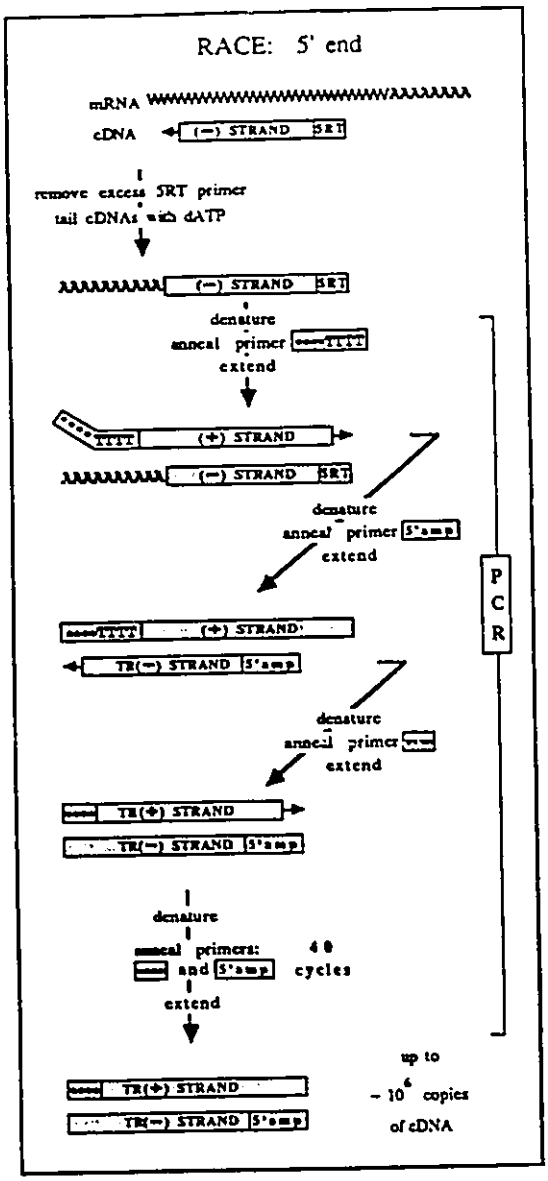



Fig. 4.2.4. The primer extension strategy "rapid amplification of cDNA ends" (RACE) used to complete the 5'-end of the thiolase 2 cDNA. The 5RT primer is primer AB704, which is complementary to the (+) strand, and used to prime reverse transcription. The oligo$(dT)_{17}$-adaptor (****TTTT) is primer AB703 which has the sequence 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'containing *Xho*I, *Sal*I and *Cla* I restriction sites. The 5'amp primer is primer AB616, complementary to the (+) strand and located farther upstream of the 5RT primer for increased specificity. The adaptor primer (****) is primer AB705 which has the sequence 5'-GACTCGAGTCGACATCG-3'and was used for the PCR amplification of cDNA. The open rectangles represent DNA strands actively being synthesized; shaded rectangles represent DNA previously synthesized. The (-) or (+) strand is indicated. Reproduced from Frohman *et al* (1988).

For this purpose, a number of synthetic oligodeoxyribonucleotides were synthesized complementary to the 5'-end of the thiolase 2 partial recombinant (pT4-4) within regions that are divergent between the thiolase 1 and 2 cDNAs (Materials and Methods, section 2.1.7, Table 2.1.1). One primer (AB704, Fig. 4.2.3, open box) was used to direct first-strand cDNA synthesis from rat liver poly(A)$^+$RNA. The products of this reaction were analyzed on a 4% polyacrylamide denaturing gel. The major product was a 480 nucleotide fragment (Fig. 4.2.5, panel A). The first strand of cDNA was then tailed using dATP and terminal deoxynucleotidyl transferase. The second strand cDNA synthesis was primed with the $(dT)_{17}$-adaptor consisting of oligo-dT and a stretch of nucleotides containing several rare restriction endonuclease sites (*Xho*I, *Sal*I and *Cla*I). Polymerase chain reaction (PCR) amplification was employed to generate multiple copies of the cDNA using primers AB616, which binds upstream of the reverse-transcriptase primer within the known sequence of the thiolase 2 cDNA, and primer AB705, which binds to the region complementary to the adaptor at the very 5'-end of the newly synthesized cDNA. Following PCR amplification, the resultant cDNA products were resolved by agarose gel electrophoresis. A band of approximately 450 bp was observed (Fig. 4.2.5, panel B). The specificity of the reaction was confirmed by Southern blot analysis with another primer made specific to the thiolase 2 cDNA sequence (primer AB755, Fig. 4.2.5, panel C). The resultant cDNA products

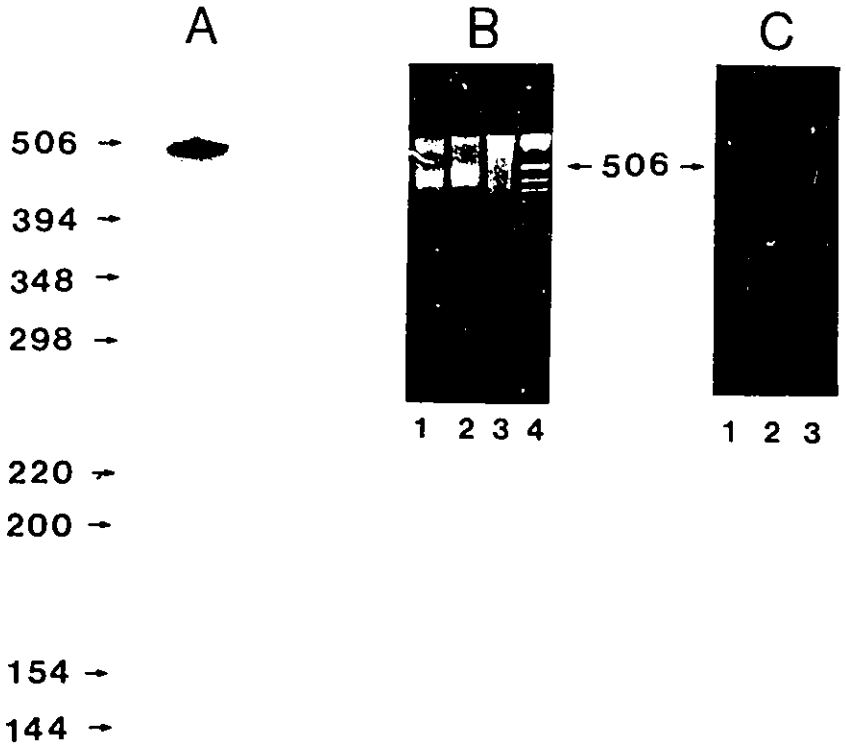



Fig. 4.2.5. Products of the RACE primer extension procedure. Panel A: autoradiogram of the $^{32}$P-labeled first-strand of cDNA synthesized by primer extension using the RT primer (AB704) resolved on a 4% polyacrylamide denaturing gel. The molecular size standards (in nucleotides) are indicated at the left. Panel B: the PCR reaction products were electrophoresed in a 4% agarose gel. Lanes 1 and 2, products of two PCR reactions using different starting preparations of RNA; lane 3, control reaction, identical reaction conditions as in lane 2 but without specific 5'amp primer (AB616); lane 4, molecular size standards. The sizes are identical to those marked in panel A (in bp), but only the 506 bp marker is labeled. Panel C: Southern blot of PCR reaction products probed with primer AB755. Primer AB755 binds to the thiolase 2 cDNA in a region upstream of the 5'amp primer.

were digested with *Sst*II (which cuts within the known thiolase 2 cDNA sequence) and *Sal*I (the *Sal*I site is present in the adaptor at the 5'-end of newly synthesized cDNA) and were subcloned into pGEM-5Zf(+). Plasmids pT5'-5 and pT5'-6 (Fig. 4.2.3) containing cDNA inserts from two independent PCR reactions were selected by colony hybridization with primer AB755, and their cDNA inserts were sequenced. The partial restriction map, primer-extended region and sequencing strategy for thiolase 2 cDNA are presented in Fig. 4.2.3.

The complete nucleotide sequence for the thiolase 2 cDNA and the deduced amino acid sequence are depicted in Fig. 4.2.6. There is a single open reading frame of 1302 nucleotides which encodes a protein of 434 amino acids ($M_r$ 44 790). The proposed ATG start codon shown at position +1 to +3 lies within a suitable consensus sequence for translation initiation by eukaryotic ribosomes (Kozak, 1986). An in-frame TAA stop codon upstream from this ATG, at nucleotide position -15 to -13, is doubly-underlined.

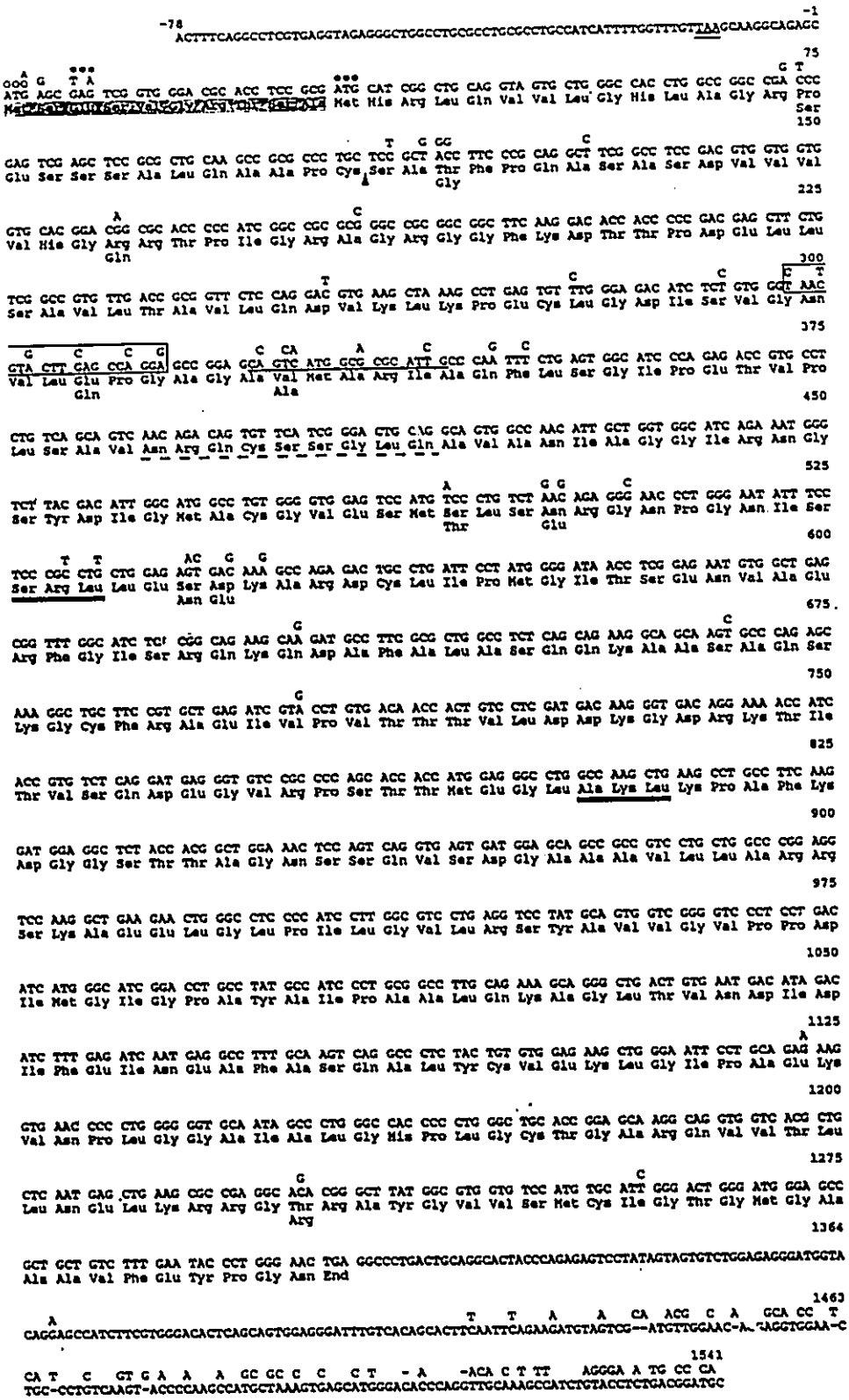
## 4.3 Comparison of the Nucleotide Sequences of cDNAs and Deduced Amino Acid Sequences of Rat Peroxisomal Thiolases 1 and 2

Comparisons of the nucleotide sequences of the thiolase 1 and 2 cDNAs and deduced amino acid sequences are shown in Fig. 4.2.6. The complete nucleotide sequence for the thiolase 2 cDNA and its deduced amino acid sequence are shown

in full. Differences in the thiolase 1 cDNA and deduced amino acid sequence are indicated above and below the sequence, respectively. The coding regions exhibit 94.6% nucleotide sequence identity, while the 3′-noncoding regions are more divergent, exhibiting 74.8% nucleotide sequence identity. The 5′-noncoding regions could not be compared as the thiolase 1 cDNA possesses only 28 nucleotides in its 5′-noncoding region. Hijikata *et al* (1987) determined 25 nucleotides of 5′-noncoding region of thiolase 1 cDNA. More recently, Hijikata *et al* (1990) added an additional 3 nucleotides of sequence to the 5′-noncoding region of the thiolase 1 cDNA sequence, and determined this to be the transcription initiation site. The ATG marked with solid dots represents the start codon for thiolase 1 (Hijikata et al, 1987). Upstream from this ATG, an in-frame TAA stop codon (indicated with asterisks) exists in the thiolase 1 cDNA but not in the thiolase 2 cDNA sequence. Therefore, it is proposed that translation of thiolase 2 is initiated from the most 5′ start codon (ATG marked by open circles), which generates an additional ten amino acids at the amino-terminus of thiolase 2 relative to thiolase 1. This ATG possesses a more appropriate consensus sequence for translation initiation by eukaryotic ribosomes (Kozak, 1986) than the second in-frame ATG.

A comparison of the deduced amino acid sequences of rat peroxisomal thiolase 2 and thiolase 1 revealed an overall

Fig. 4.2.6. Composite nucleotide sequence and deduced amino acid sequence of the thiolase 2 cDNA and comparison with thiolase 1. Nucleotides are numbered with the first nucleotide of the putative start codon of thiolase 2 as +1; nucleotides in the 5′-untranslated region are denoted by negative numbers. The complete nucleotide sequence for the thiolase 2 cDNA and its deduced amino acid sequence are shown in full. Differences in the thiolase 1 cDNA are indicated above the thiolase 2 sequence. Differences in the deduced amino acid sequence of thiolase 1 are indicated below. Gaps (-) are inserted in the sequence to obtain maximum identity. The putative start codon for thiolase 2 is marked with open circles. The doubly-underlined nucleotide sequence represents an in-frame TAA stop codon upstream from the Met start codon of thiolase 2. Solid dots mark the start codon for thiolase 1. Upstream from this ATG is an in-frame TAA stop codon (indicated with asterisks) which is present in the thiolase 1 cDNA but absent in the thiolase 2 cDNA. This generates an additional 10 amino acids at the amino-terminus of thiolase 2 relative to thiolase 1 (shaded region). The 5′-end of the thiolase 2 cDNA was obtained by primer extension of mRNA using a primer complementary to the sequence underlined with a thin line (AB704). The divergent region between thiolase 1 and thiolase 2 (boxed) is the sequence to which oligodeoxyribonucleotide probes were synthesized for northern blot analyses (AB615 and AB616). The tripeptides underlined with a thick line represent variants of the proposed PTS. The upward arrowhead indicates the site of proteolytic processing of thiolase 1. The putative substrate-binding region is underlined with a dashed line. Computer analysis was performed with the Beckman MICROGENIE software package (Queen and Korn, 1984).



identity of 95.4%, the most significant difference being an additional ten amino acids at the amino-terminus of native thiolase 2 (Fig. 4.2.6, shaded region). There are ten mismatches in the remaining sequence, two of which are conservative changes. The putative substrate binding region is underlined with a dashed line (Fig. 4.2.6). The consensus sequence, Asn-Xaa$^1$-Xaa$^2$-Cys-Xaa$^3$-Ser-Gly-Xaa$^4$-Xaa$^5$, where Xaa$^1$ is a basic residue, Xaa$^3$ is Gly, Ala or Ser, Xaa$^4$ is hydrophobic and Xaa$^2$ and Xaa$^5$ are glutamines, is typical of a 3-ketoacyl-CoA thiolase (Fukao et al, 1989). The consensus sequence for an acetoacetyl-CoA-specific thiolase contains a basic residue in position Xaa$^5$ (Fukao et al, 1989). For this reason we suggest that thiolase 2 is a second peroxisomal 3-ketoacyl-CoA thiolase.

It is interesting to note that the 5′-regions of the thiolase cDNAs are less conserved than the 3′-regions. If we assume that these two genes arose from a common ancestral gene (discussed in section 4.6), this 5′-region may be less conserved because it is not important in enzyme function or because the two forms of the enzyme may have evolved to acquire different functions by accumulating mutations in this region (Miyazawa *et al*, 1987).

## 4.4 Northern Blot Analysis of mRNAs Encoding Thiolase 1 and Thiolase 2

Poly(A)$^+$ RNA was isolated from the livers of untreated and clofibrate-treated rats and probed with oligodeoxyribonucleotides specific for thiolase 1 or thiolase 2 mRNA (Fig. 4.4.1). The mRNAs encoding thiolases 1 and 2 were similar in size (approximately 1.7 kb) but showed very different patterns of induction upon treatment of rats with clofibrate. The thiolase 2 mRNA was present in normal rat liver and induced about two-fold upon treatment of the rats with clofibrate (Fig. 4.4.1, thiolase 2, compare lane U to lane C). However, the thiolase 1 message was present in very low amounts (not detected on this northern blot) in untreated rat liver and was induced greater than ten-fold upon clofibrate treatment (Fig. 4.4.1, thiolase 1, compare lane U to lane C). The levels of induction of thiolase 1 and 2 mRNAs were quantitated using RNA slot blot analysis (Fig. 4.4.2). Poly(A)$^+$RNA samples were applied to nitrocellulose in step-wise decreasing increments and the blots were treated with $^{32}$P-labeled oligodeoxyribonucleotide probes specific for thiolase 1 or 2 mRNA (Fig. 4.4.2, top panels). The hybridization and wash conditions were identical to those used in northern blot analysis so that only one specific message was detected by each probe. To standarize the slot blots, the probe was boiled off the blots, and the amounts of RNA bound to the nitrocellulose were quantitated by binding of radiolabeled

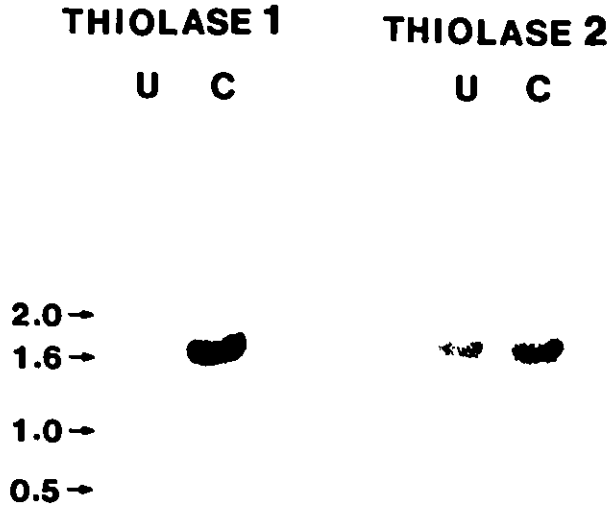



Fig. 4.4.1. Northern-blot analysis of rat liver poly(A)$^+$RNA. Poly(A)$^+$RNA was isolated from livers of untreated (U) and clofibrate-treated (C) rats and analyzed by northern blots (5 μg per lane). The blots were probed with $^{32}$P-labeled oligodeoxyribonucleotides specific for thiolase 1 or thiolase 2 mRNA (primers AB615 and AB616, respectively, synthesized complementary to the boxed region in Fig. 4.2.6). DNA markers are indicated on the left margin in kb.

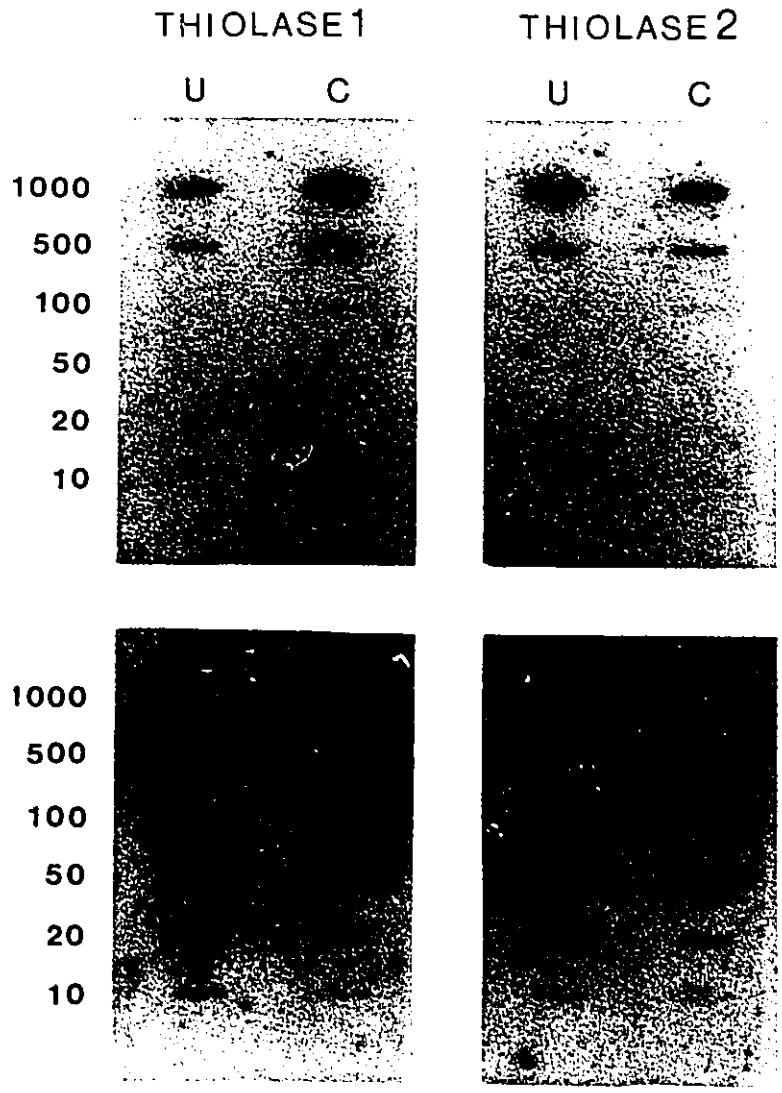



Fig. 4.4.2. Slot-blot analysis of liver poly(A)$^+$ RNA from untreated and clofibrate-treated rats probed with oligodeoxyribonucleotides specific for thiolase 1 or thiolase 2 mRNA. Poly(A)$^+$ RNA isolated from untreated (U) and clofibrate-treated (C) rat livers was applied to nitrocellulose in step-wise decreasing increments. The number of nanograms of poly(A)$^+$ RNA loaded in each well is indicated at the left. The upper blots were probed with $^{32}$P-labeled oligodeoxyribonucleotides specific for thiolase 1 or thiolase 2 mRNA (primers AB615 and AB 616, respectively, synthesized complementary to the boxed region in Fig. 4.2.6). In the bottom two blots the radiolabeled probe was boiled off the blots and the blots were reprobed with $^{32}$P-labeled oligo(dT)$_{18}$ for quantitation.

oligo$(dT)_{18}$ (Harley, 1987; Fig. 4.4.2, bottom panels). Assuming that treatment with clofibrate did not effect the length of the poly(A)$^+$ tails of mRNA, this is a valid method of quantitation. The levels of induction were quantitated by densitometric analysis of the resultant autoradiograms. After correcting for errors resulting from unequal amounts of mRNA in adjacent lanes of the slot blot, the induction of thiolase 1 mRNA was 13.1 ± 2.1-fold (n=4) upon clofibrate treatment, whereas thiolase 2 mRNA was induced only 2.5 ± 0.3-fold (n=4) under similar conditions. The existence of the two differentially regulated forms of thiolase makes thiolase a good model system for investigating the induction of expression of peroxisomal genes by hypolipidemic drugs such as clofibrate.

Hijikata *et al* (1990) have characterized two closely related but differentially regulated genes for rat peroxisomal 3-ketoacyl-CoA thiolase (B and A) which correspond to the cDNAs encoding thiolases 1 and 2, respectively. The high level of identity (>97%) between the nucleotide sequences of the coding regions and even between some of the 11 introns (introns 1, 4, 8, 9, 10 and 11 are highly conserved, exhibiting >89% sequence identity) suggests that the two genes arose by a recent gene duplication event. The remaining noncoding regions and the flanking regions have diverged more over this time. It is the divergent 5'-flanking regions which are thought to be responsible for the differential regulation

of expression of the two peroxisomal thiolases. The 5'-flanking region of the inducible thiolase 1 (B) gene has three short sequence motifs (9-10 bp each) in common with the 5'-flanking regions of the clofibrate-inducible genes for peroxisomal fatty acyl-CoA oxidase and hydratase dehydrogenase (Hijikata *et al*, 1990). These motifs represent putative peroxisome proliferator-responsive elements (Hijikata *et al*, 1990).

There is only one gene for rat liver peroxisomal fatty acyl-CoA oxidase, but it gives rise to two mRNAs by alternative splicing (Osumi *et al*, 1987; Schepers *et al*, 1990). These two mRNAs encode two slightly different protein species, an "inducible" form of fatty acyl-CoA oxidase and a "noninducible" form of fatty acyl-CoA oxidase. This leads to the interesting situation in which the rate of transcription of a single acyl-CoA oxidase gene is increased upon treatment with peroxisome proliferators but the concentration of only one of the two alternatively spliced mRNAs is increased. This leaves us with the question of why these two mechanisms (gene duplication and alternative splicing) have evolved to produce an inducible and a non-inducible form of peroxisomal thiolase and fatty acyl-CoA oxidase.

## 4.5 Identification of the Initiator Methionines for Thiolases 1 and 2

The observation that there was an abundance of

thiolase 2 message in RNA from untreated rat liver and an abundance of thiolase 1 message in RNA isolated from liver of clofibrate-treated rats allowed tentative identification of the start codons used by the two thiolases. mRNAs from untreated and clofibrate-treated rat livers were translated *in vitro*, and the translation products were immunoprecipitated with anti-thiolase serum (Fig. 4.5.1). Two thiolases of different molecular masses were immunoprecipitated from the translation products of RNA from untreated liver; however, the higher molecular mass form predominated. The predominant thiolase species immunoprecipitated from the translation products of RNA from clofibrate-treated liver was the lower molecular mass species. We propose that this difference in molecular mass is due to the additional ten amino acids present at the amino-terminus of thiolase 2 (more abundant in the translation products of RNA from untreated liver) relative to thiolase 1 (more abundant in the translation products of RNA from clofibrate-treated liver), which is consistent with the two thiolases initiating translation from different ATG start codons, at least *in vitro*. The newly synthesized thiolases migrated higher in SDS-PAGE than the mature thiolases isolated from rat liver peroxisomes, indicating that both thiolase 1 and 2 are proteolytically processed.

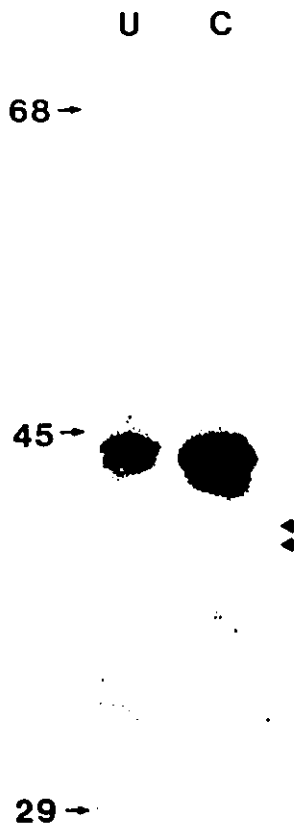
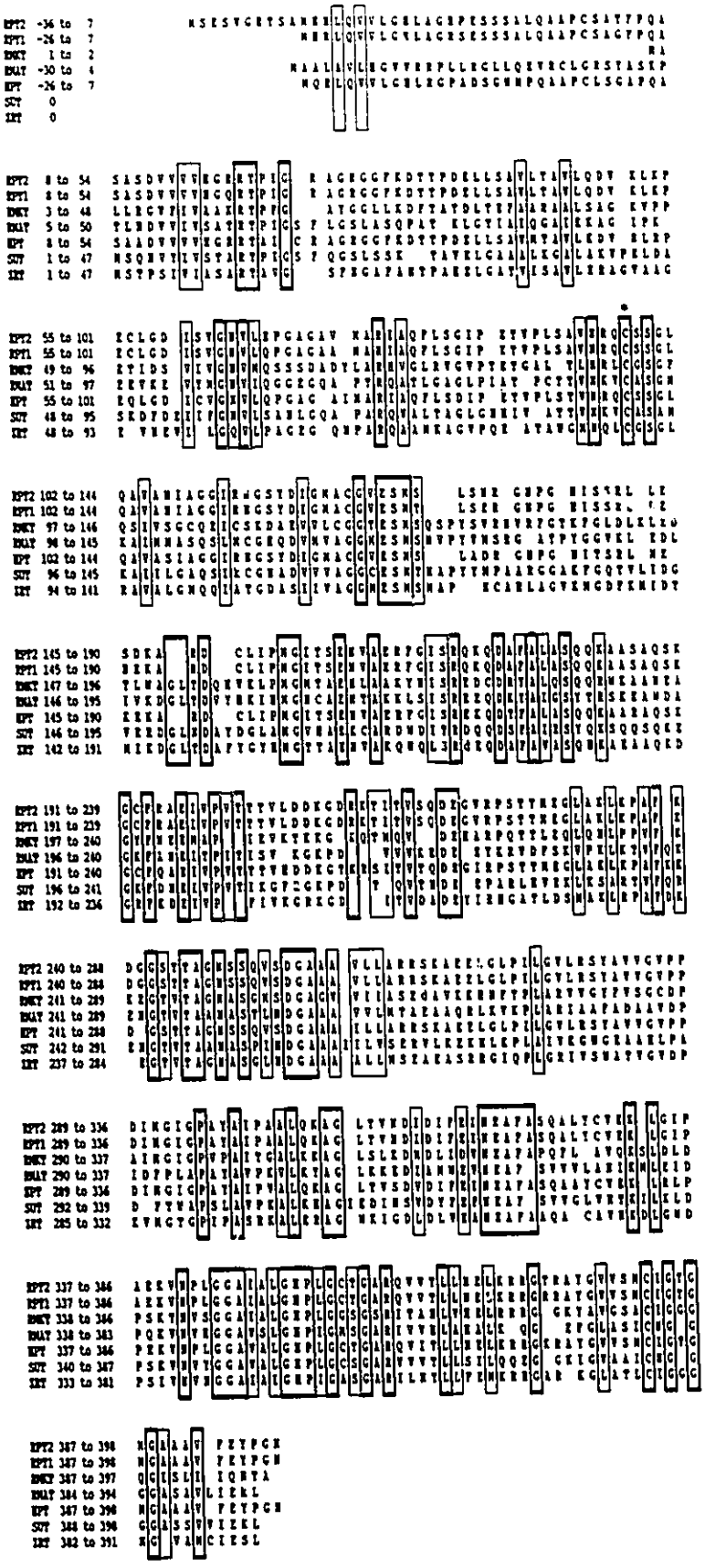



Fig. 4.5.1. Fluorogram of cell-free translation products of RNA isolated from untreated (U) and clofibrate-treated (C) rat liver immunoselected with anti-thiolase serum. Cell-free translations were performed in rabbit reticulocyte lysates with 1.0 $\mu$g of poly(A)$^+$RNA from untreated or clofibrate-treated rat liver. $4 \times 10^6$ cpm of TCA-precipitable counts from translation mixtures programmed with RNA from untreated liver or $6 \times 10^5$ cpm of TCA-precipitable counts from the mixtures programmed with RNA from clofibrate-treated liver were immunoprecipitated with anti-thiolase serum. The immunoprecipitates were separated by SDS-PAGE followed by fluorography. The arrowheads represent the two mature peroxisomal protein bands that react with anti-thiolase serum. Markers (in kDa) are indicated on the left margin.

## 4.6 Comparison of Amino Acid Sequences of Rat Peroxisomal Thiolases 1 and 2 With Other Thiolases

Several thiolases have been reported in mammalian cells. A cytosolic acetoacetyl-CoA thiolase is required for cholesterol biosynthesis (Fukao *et al*, 1989). Mitochondria possess two different thiolases, an acetoacetyl-CoA thiolase, which probably functions in ketone body metabolism, and a 3-ketoacyl-CoA thiolase, which catalyzes the final step of mitochondrial ß-oxidation (Fukao *et al*, 1989). In addition, two peroxisomal 3-ketoacyl-CoA thiolases have been implicated in peroxisomal ß-oxidation (Bodnar and Rachubinski, 1990), and recently an acetoacetyl-CoA thiolase activity has been localized to rat liver peroxisomes which may play a role in cholesterol biosynthesis (Thompson and Krisans, 1990). The cytosolic, mitochondrial and peroxisomal thiolases all differ in both molecular and catalytic properties; however, comparison of the amino acid sequences of known thiolases reveals marked pair identity (34 to 95.4%) (Fukao *et al*, 1989; Bodnar and Rachubinski, 1990). Figure 4.6.1 illustrates an amino acid alignment among the thiolases whose sequences are known, including a sequence for acetoacetyl-CoA thiolase of *Zoogloea ramigera* and that for cytosolic acetoacetyl-CoA thiolase of *Saccharomyces uvarum* (Peoples *et al*, 1987; Dequin *et al*, 1988). The high degree of identity between the different thiolases suggests that they share a common ancestral gene. Therefore they must have obtained information

Fig. 4.6.1. Comparison of amino acid sequences of rat peroxisomal thiolases 1 and 2 with other thiolases. The thiolases are: rat peroxisomal 3-ketoacyl-CoA thiolase 2 (RPT2) and thiolase 1 (RPT1), rat mitochondrial 3-ketoacyl-CoA thiolase (RMKT), rat mitochondrial acetoacetyl-CoA thiolase (RMAT), human peroxisomal 3-ketoacyl-CoA thiolase (HPT), cytosolic acetoacetyl-CoA thiolase of *Saccharomyces uvarum* (SUT) and acetoacetyl-CoA thiolase of *Zoogloea ramigera* (ZRT). The one-letter amino acid code is used, and gaps are inserted to obtain maximum identity. Amino acid residues are numbered such that the first amino acids of the mature enzymes are +1, and amino acids in the cleaved presequences are denoted by negative numbers. The amino acid residues outlined by double boxes indicate an alignment of identical residues, those in single boxes indicate chemically similar residues. The cysteine residue indicated by an asterisk is the putative substrate binding site involved in the formation of an acyl-S-enzyme intermediate.



for targeting to their respective intracellular compartments, as well as for substrate specificity, during the course of evolution (Fukao *et al*, 1989). It is interesting to note that the amino-terminal regions of the thiolases are less conserved than the carboxy-terminal regions. This observation might indicate that each thiolase has acquired specific sequences or structures for intracellular location and different substrate specificity within the amino-terminal portion during its evolution. From this type of multiple sequence alignment the degree of evolutionary relationship among family members can be inferred. An evolutionary tree was constructed using the CLUSTAL program from the PC gene software package (Higgins and Sharp, 1988). It was constructed by the method referred to as unweighted pair group maximum averages (UPGMA) which begins by constructing a matrix of all pairwise sequence similarities and then progressively aligns the most similar sequences. This analysis indicated that the gene that gave rise to peroxisomal thiolase and that which gave rise to the mitochondrial thiolase first diverged from a common ancestral gene. The peroxisomal thiolase, which has broad substrate specificity, later diverged to give rise to the genes for peroxisomal thiolases 1 and 2. The gene for acetoacetyl-CoA-specific thiolase diverged from the gene for mitochondrial 3-ketoacyl-CoA thiolase. This scheme is in agreement with that derived by Fukao *et al* (1989) using sequences of 5 thiolases; however, further expansion and verification of this

evolutionary tree await analysis of the genes and/or proteins of other members of this multigene family. The UPGMA method is only one of a number of methods from which to choose, and although powerful in its own right, it has been criticized because of its inability to deal with unequal rates of evolution along different lineages, which can lead to errors in branching order (Higgins and Sharp, 1988). Keeping in mind the limitations of the package, the UPGMA method serves as a good starting point for discussion.

## 4.7 In Vivo Targeting Studies of Peroxisomal Thiolase in Mammalian Cells

### 4.7.1. The Role of the Prepiece in Targeting Thiolase to Peroxisomes

There are many potential functions of the prepiece located at the amino-terminus of peroxisomal thiolase. It has been suggested that the prepiece may mask a mitochondrial targeting signal, or it may in itself contain targeting information for directing this protein to peroxisomes (Borst, 1986; Arakawa *et al*, 1987). In contrast, the prepiece may not contain targeting information but could interfere with tight folding of the protein, keeping it in an import competent state, or could play a role in a posttranslational control system (Borst 1986). The cloning of rat liver peroxisomal thiolase cDNA was undertaken primarily to faciltate studies investigating the role, if any, of the presequence in

targeting this protein to peroxisomes. The approach that was used to investigate this was an *in vivo* import assay developed by Gould *et al* (1987; 1988; 1989). This involved subcloning the thiolase cDNA and mutated forms of the thiolase cDNA into a mammalian expression vector and transfecting monkey kidney cells. The location of the newly synthesized thiolase was evaluated by immunofluorescence microscopy. The results of these experiments were included to complete the discussion; however the plasmid constructions, transfections and immunofluorescence microscopy were performed by Dr. B.W. Swinkles and Dr. S.J. Gould in the laboratory of Dr. S. Subramani (Dept. of Biology, University of California at San Diego). We supplied two plasmids, one containing the cDNA for mature thiolase 2 (pT4-4) and the other encoding the prepiece of thiolase 2 (pT5'-6), as well as the anti-thiolase antibodies used in these experiments.

Beginning with the two partial recombinants, pT4-4 and pT5'-6, a full-length cDNA encoding the thiolase 2 precursor was constructed. Using this cDNA which encodes the larger of the two peroxisomal thiolases, an artificial thiolase 1 cDNA was constructed by deletion of the initiation codon of the thiolase 2 cDNA. This cDNA construct directed the initiation of protein synthesis from the second methionine codon in thiolase 2, which corresponds to the initiation codon of thiolase 1. The plasmid pRSV-An was used for the expression of thiolase gene constructs. Expression was directed from the

Rous sarcoma virus long terminal repeat, and the plasmid contains sequences derived from an SV40 fragment containing the early region polyadenylation signal (An) (positions 2770-2533). Between the RSV promotor and the SV40 An signal, pRSV-An contains several restriction sites for cloning.

The recombinant plasmids were introduced into CV-1 or CVH Px110 monkey kidney cell lines by calcium phosphate-mediated transfection (Parker and Stark, 1979). The CVH Px110 cell line has been engineered to express the artificial peroxisomal protein CAT-PMP20, which contains the bacterial protein chloramphenicol acetyltransferase (CAT) linked to the carboxy-terminal 12 amino acids of the peroxisomal integral membrane protein 20 from *Candida boidini* (Gould *et al*, 1990a). This fusion protein can be detected using a mouse monoclonal anti-CAT antibody. Immunofluorescent analysis of this cell line with mouse anti-CAT antibody exhibits a punctate pattern characteristic of peroxisomes. This cell line facilitates double-label immunofluorescence experiments to determine the subcellular distribution of the transiently expressed proteins. Immunofluorescence of transfected cells expressing thiolase 2 using anti-thiolase serum showed punctate patterns that were superimposable over immunoflourescence patterns with anti-CAT serum in CAT-PMP20 expressing cells. Therefore thiolase 2 was co-localized with CAT-PMP20 in peroxisomes (Fig. 4.7.2, panels A and B). As a control, thiolase was not detected in the other cell in panel A. This was probably an

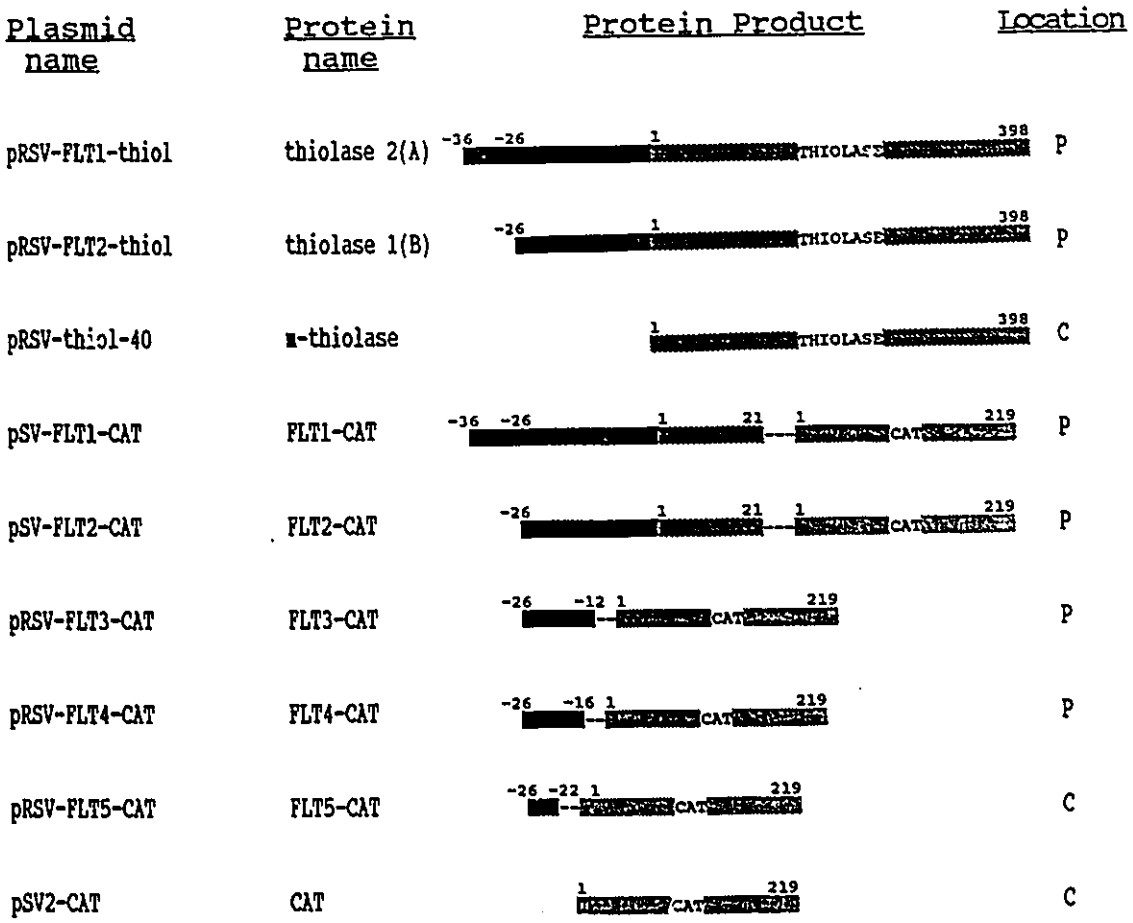



Fig. 4.7.1. Representations of the protein products encoded by the thiolase and thiolase-CAT fusion gene constructs. The black bars represent the thiolase prepieces, shaded bars represent mature thiolase or CAT sequences as indicated. The amino acid residues are numbered such that the first amino acids of the mature enzymes are +1 and amino acids in the cleaved presequences are denoted by negative numbers. The lengths of the bars are not to scale. The subcellular locations of the gene products, as determined by immunofluorescence, are indicated at the right (P=peroxisomal, C=cytosolic).

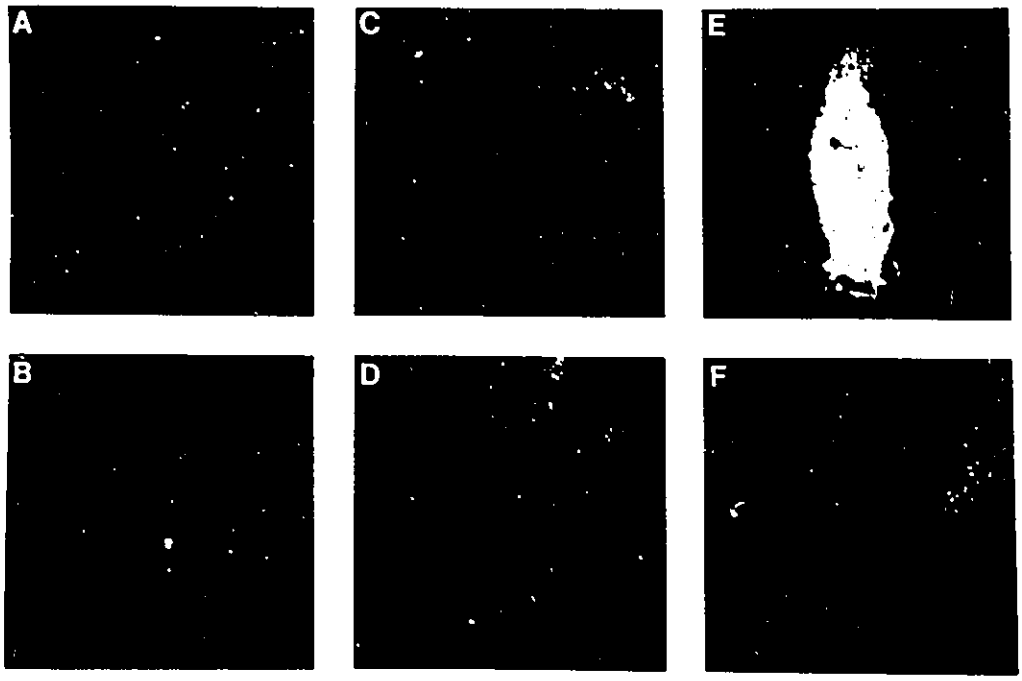



Fig. 4.7.2. Immunofluorescent localization of thiolase 2 and thiolase 1 precursors and mature thiolase in CV-H Px110 cells. CV-H Px110 cells were transfected with one of pRSV-FLT1-thiol (panels A and B), pRSV-FLT2-thiol (panels C and D) or pRSV-thiol-40 (panels E and F) encoding rat thiolase 2 , thiolase 1 and mature thiolase, respectively. Cells were processed for double labeling immunofluorescence with rabbit anti-rat peroxisomal thiolase serum and with mouse anti-CAT monoclonal antibodies to detect the peroxisomal CAT-PMP20 fusion protein. Secondary antibodies used were a rhodamine conjugate of a goat anti-mouse IgG antibody and a fluorescein conjugate of goat anti-rabbit IgG antibody. The upper panels represent the distribution of thiolase 2 (panel A), thiolase 1 (panel C) and mature thiolase (panel E), while locations of peroxisomes are indicated by the location of the CAT-PMP20 fusion protein in panels B, D and F.

untransfected cell and showed that the anti-rat thiolase serum did not react with endogenous thiolase in CV-1 cells due to differences in antigenicity or perhaps low levels of expression of endogenous thiolase. Panels C and D of Fig. 4.7.2. show that thiolase 1 is similarily located in peroxisomes of CVH Px110 cells. A construct was made in which the segment encoding the presequence was deleted and protein synthesis was programed to initiate at a position corresponding to the first amino acid of mature thiolase (Fig. 4.7.1, construct pRSV-thiol-40). Mature thiolase was not directed to peroxisomes, as punctate structures were not observed but a general cytosolic staining pattern was exhibited (Fig. 4.7.2, panel E). Taken collectively, the results indicate that the prepieces of thiolase 1 and 2 appear to be required for import of thiolase to peroxisomes.

To investigate whether the prepieces were sufficient to target a normally cytosolic protein to peroxisomes, fusion constructs were made in which the cDNA encoding either the thiolase 1 or 2 prepiece was fused in-frame to the 5'-end of the coding region of the CAT gene. The first 21 amino acids of mature thiolase were included in these constructs to preserve the site of proteolytic processing in the event that proteolysis was necessary for import (Fig. 4.7.1, FLT1-CAT, FLT2-CAT). These constructs were detected with mouse anti-CAT monoclonal antibodies in CV-1 cells, which were double-labeled with a rabbit antibody directed against the previously

characterized peroxisomal tripeptide targeting sequence (anti-SKL antibody) (Gould *et al*, 1990b). A weak punctate fluorescence was observed from cells expressing these constructs (pSV-FLT1-CAT and pSV-FLT2-CAT) using the anti-CAT antibody (data not shown). In both cases, the weak fluorescence was superimposable with the peroxisomal pattern obtained using the anti-SKL antibodies. Therefore it appeared that the thiolase presequences added to the amino-terminus of CAT were sufficient to direct CAT into peroxisomes. The poor detection of the FLT1-CAT and FLT2-CAT fusion proteins may be due to poor expression and/or detection. Perhaps the extra sequences on the CAT amino-terminus interfered with recognition by the anti-CAT monoclonal antibody. This is consistent with the observation that CAT was easily detected when much shorter sequences were fused onto the CAT amino-terminus (Section 4.7.2).

### 4.7.2 Deletion Analysis of the Thiolase Prepiece

Deletion analysis of the thiolase prepiece was undertaken in an attempt to define the minimal sequence requirement that was still capable of directing proteins to peroxisomes. Constructs were made which encoded the first 15, 11 or 5 amino acids of the thiolase 1 prepiece fused onto the amino terminus of CAT (Fig. 4.7.1, FLT3-CAT, FLT4-CAT, FLT5-CAT). In Fig. 4.7.3, panels A and B and panels C and D show that the first 15 or 11 amino acids of the thiolase prepiece,

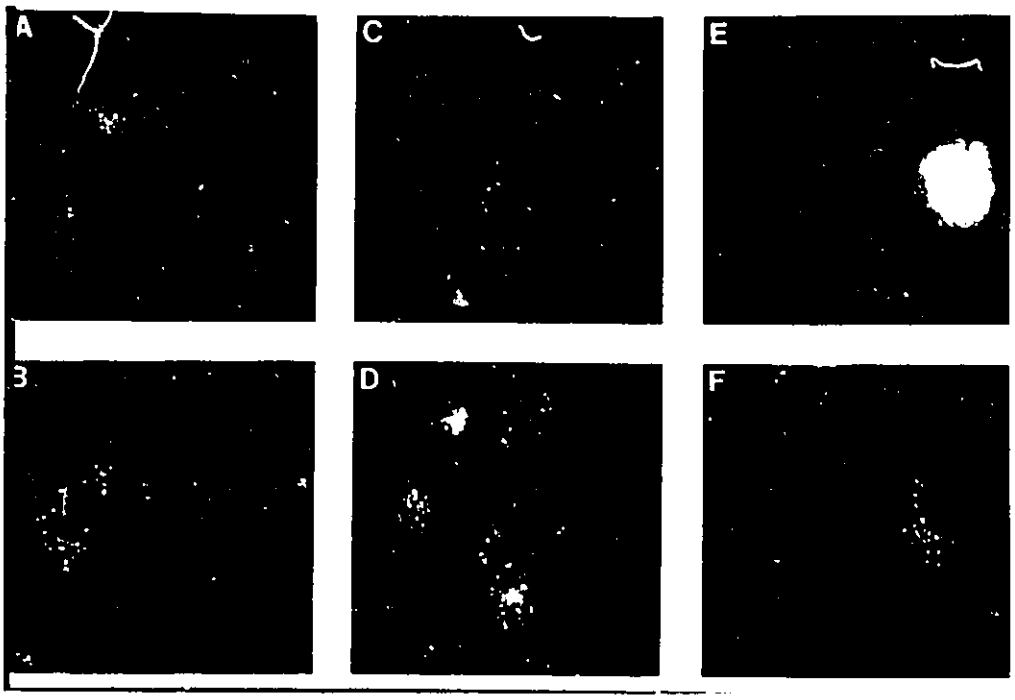



Fig. 4.7.3. Immunofluorescent localization of thiolase-CAT fusion proteins containing prepiece deletions. CV-1 cells were transfected either with pRSV-FLT3-CAT (panels A and B), pRSV-FLT4-CAT (panels C and D) or pRSV-FLT5-CAT (panels E and F). Cells were processed for double label immunofluorescence using mouse anti-CAT monoclonal antibodies to detect the thiolase-CAT fusion proteins and rabbit anti-SKL antibodies to stain peroxisomes. Secondary antibodies used were a fluorescein conjugate of a goat anti-mouse IgG antibody and a rhodamine conjugate of a goat anti-rabbit IgG antibody. The upper panels show the distribution of FLT3-CAT (panel A), FLT4-CAT (panel C) and FLT5-CAT (panel E), while the bottom panels show the location of peroxisomes (panels B, D and F).

respectively, were sufficient to target CAT to peroxisomes. The general cytosolic staining for construct FLT5-CAT illustrated that the five amino acid prepiece was unable to target CAT to peroxisomes (Fig. 4.7.3, panel E). From this we concluded that the first 11 amino acids of the thiolase 1 prepiece either constitute or contain a novel PTS.

This novel PTS is located at the amino-terminus of rat peroxisomal thiolase 1 and does not contain a tripeptide that conforms to the consensus sequence of the carboxy-terminal tripeptide PTS. Interestingly, this targeting signal is not situated at the extreme amino-terminus of the prepiece of thiolase 2 but is preceded by 10 amino acids. Therefore, this novel PTS is functional at an internal location. It will be of interest to determine how many amino acids can precede this PTS without affecting its function or if it can function at the carboxy-terminus.

The occurence of this novel PTS implies that there may be more than one distinct pathway for targeting proteins to the peroxisome. Additional evidence for this comes from studies conducted using cells from patients with Zellweger syndrome. Recently, Santos *et al* (1988a; 1988b) found that cells from Zellweger syndrome patients contain peroxisomal "ghosts", membrane vesicles containing the peroxisomal IMPs but which appear to lack the matrix proteins. More recently, Balfe *et al* (1990) have found that in some cases of this peroxisomal disorder, the thiolase precursor is associated

with these peroxisomal "ghosts", indicating that thiolase is imported into peroxisomes by a pathway independent of that used by most of the peroxisomal matrix proteins. Thus, in mammalian cells, there appear to be at least two independent pathways for import of proteins into peroxisomes. It will be of interest to determine if thiolase and the IMPs use the same import machinery or whether these are two separate pathways, suggesting the existence of multiple pathways for peroxisomal protein import. This diversity in transport pathways is not unprecedented, as both mitochondria and the ER have been shown to possess more than one possible targeting pathway. In mitochondria, the existence of multiple import pathways was shown by competition between different precursor proteins for uptake into isolated mitochondria (Zimmerman, 1981). These studies illustrated that certain precursors can effectively compete for uptake of some precursors but not others (Zimmerman, 1981). Similar results were obtained from experiments showing that a synthetic peptide with a motif common to the prepiece of several mitochondrial precursor proteins blocks the uptake of these precursors into isolated mitochondria; however, precursors without this motif are not affected (Yoshida *et al*, 1985). In addition, there are examples of proteins targeted to the ER that do not use the traditional ER transport machinery (Pugsley, 1989; Powers *et al*, 1986; Rachubinski *et al*, 1980; Borgese and Gaetani, 1980).

The existence of multiple transport pathways seems

redundant, but it may increase the flexibility or diversity of import (Borst, 1989). For example, one targeting signal might not be compatible with the function or stability of all peroxisomal proteins (Borst, 1989). It is important to keep in mind that these pathways are not necessarily completely independent from one another and that many components of the peroxisomal import pathways may be conserved. For example, the cytosolic factors may be conserved, but there may exist different membrane associated receptors for different classes of proteins, as appears to be the case for mitochondria (Baker and Schatz, 1991).

### 4.7.3 Characteristics of the Thiolase Prepiece

A search of the Genetic Sequence Data Bank (GenBank) and the National Biomedical Research Foundation (NBRF) Protein Identification Resource Data Bank found no significant homology between the thiolase prepiece and other known proteins. In particular we looked for similarity between the thiolase prepiece and the prepieces of other microbody proteins that are known to be processed (Table 4.7.1). Of the proteins listed in Table 4.7.1, sequences are available for rat 3-ketoacyl-CoA thiolases 1 and 2, human 3-ketoacyl-CoA thiolase, watermelon malate dehydrogenase (MDH) and human sterol carrier protein 2 (SCP2). There is high sequence homology between the rat thiolase presequences and the human thiolase presequence showing evolutionary conservation between

Table 4.7.1. Microbody Proteins Synthesized As Detectable Precursors

| Protein | Reference |
|---|---|
| 3-ketoacyl-CoA thiolase (rat, human liver) | Fujiki et al, 1985; Bout et al, 1988 |
| malate dehydrogenase (watermelon, cucumber) | Riezman et al, 1980; Gietl, 1990 |
| catalase (pumpkin, cucumber) | Riezman et al, 1980; Yamaguchi et al, 1984 |
| carnitine acetyltransferase (71 kDa subunit, C. tropicalis) | Ueda et al, 1984 |
| sterol carrier protein 2 (rat, human liver) | Fujiki et al, 1989; Yamamoto et al, 1991 |

these two species. However, there is no significant sequence homology between the thiolase prepiece and the prepieces of the other microbody proteins (Fig. 4.7.4). The rat thiolase 1 prepiece exhibits only 27% and 15% sequence identity with the watermelon MDH prepiece and the human SCP2 prepiece, respectively. It is not surprising that there is no direct amino acid sequence identity between these three known leader sequences, because these types of sequence homologies are often not found in the presequences of other known intracellular sorting pathways (*e.g.* mitochondria and ER). The amino-terminal presequences for ER and mitochondrial proteins are highly variable in size (13-36 amino acids) and sequence (Borst, 1986), but they share some more general properties. For example, the mitochondrial amino-terminal presequences are rich in basic amino acids, and serine and leucine residues are also relatively abundant, while acidic residues are few (Borst, 1986). These signal peptides often have the potential to form amphipathic α-helices with the positively charged residues on one side of the helix and the hydrophobic residues on the other side of the helix (Allison and Schatz, 1986). However, it is not clear if the formation of an α-helix is essential for targeting proteins to mitrochondria, as Allison and Schatz (1986) determined that this may depend simply on the overall balance between basic, hydrophobic and hydroxylated amino acids in the presequences. On the other hand, the ER amino-terminal signal possesses an

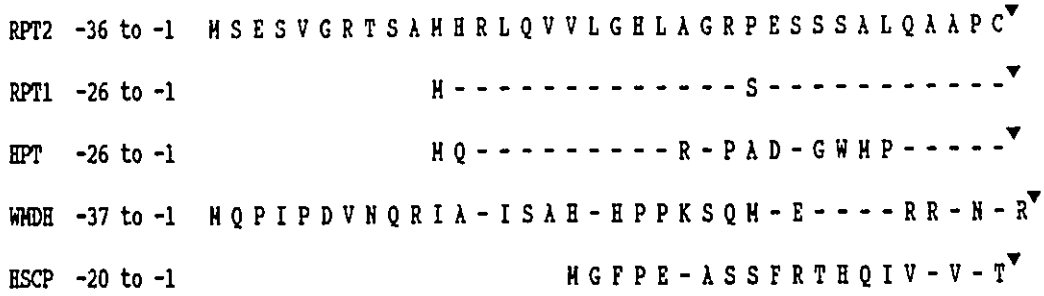



Fig. 4.7.4. Amino acid sequence comparison of the rat peroxisomal thiolase presequences with the presequences of other microbody proteins. The protein sequences are: rat peroxisomal 3-ketoacyl-CoA thiolase 2 (RPT2) and thiolase 1 (RPT1), human peroxisomal 3-ketoacyl-CoA thiolase (HPT), watermelon malate dehydrogenase (WMDH) and human sterol carrier protein 2 (HSCP). The one-letter amino acid code is used, and dashes indicate residues which are identical to rat peroxisomal thiolase 2. The amino acid residues of the presequences are numbered with negative numbers with the residue at the cleavage site denoted -1. The cleavage site is indicated by arrowheads.

amino-terminal part containing basic residues with a net postitive charge, a hydrophobic middle section (5-7 amino acids) and a less hydrophobic carboxy-terminal part (Borst, 1986). Therefore it may be more informative to look for the occurrence of the various groups of amino acids. In this respect, all peroxisomal presequences possess a high percentage of hydrophobic amino acids (32.4-42.3%), basic residues (10-21.6%) and with the exception of the human thiolase presequence, serine and threonine residues (10.8-20%) and few acidic residues (Table 4.7.2). These residues occur without any obvious pattern. There is a small stretch of amino acids which is conserved between the rat liver thiolase prepiece and the prepiece of watermelon MDH (amino acids -6 to -11 in thiolase 2 and -7 to -11, in MDH, respectively, Fig. 4.7.4). This region contains an acidic residue, followed by two or three serine residues and then by two hydrophobic residues (Ala and Leu). However, we must question the importance of this region in peroxisomal targeting, as the thiolase 1 presequence construct containing the first 11 amino acids, not including this region, was able to target proteins to peroxisomes. These 11 amino acids of the rat thiolase presequence contain 3 basic, 6 hydrophobic and no hydroxylated amino acids and do not exhibit a tendency to form an amphipathic α-helix. The corresponding region in MDH similarly has 3 basic and 5 hydrophobic residues; however, there is no obvious similarity in the arrangement of these

Table 4.7.2. Distributions of Amino Acids in the Microbody Prepieces

| Prepiece | Percent Distribution | | | |
|---|---|---|---|---|
| | Hydrophobic (A, I, L, V, M, F, W, Y) | Hydroxylated (S, T) | Basic (H,R,K) | Acidic (D, E) |
| RPT2 | 38.9% | 19.4% | 13.9% | 5.6% |
| RPT1 | 42.3% | 15.4% | 15.4% | 3.8% |
| HPT | 42.3% | 3.8% | 11.5% | 3.8% |
| WMDH | 32.4% | 10.8% | 21.6% | 8.1% |
| HSCP2 | 40.0% | 20.0% | 10.0% | 10.0% |

amino acids. It is difficult to draw conclusions from this limited comparison. Further understanding of the role of the presequences in targeting awaits firstly, confirmation that they are all involved in targeting, and secondly, mutational analysis to elucidate the critical features of each targeting signal.

## 4.8 Summary and Future Directions

To investigate further the properties of peroxisomal thiolase, several cDNA recombinants were selected using an antibody directed against this protein. The complete cDNA sequence and the deduced amino acid sequence of a second rat liver peroxisomal 3-ketoacyl-CoA thiolase (thiolase 2) were determined. The peroxisomal thiolase 2 cDNA shared 94.6% nucleotide sequence identity with the cDNA encoding thiolase 1 and 95.4% amino acid sequence identity. The most significant difference was an additional 10 amino acids at the amino-terminus of thiolase 2. Northern-blot analysis indicated that the messages encoding thiolases 1 and 2 were similar in size but showed markedly different patterns of induction upon treatment of rats with clofibrate. Thiolase 1 transcription was induced greater than ten-fold upon treatment of rats with clofibrate, whereas the thiolase 2 transcript was induced only about 2-fold under similar conditions.

Both peroxisomal thiolases 1 and 2 were synthesized with precursor extensions that are cleaved to generate the

mature enzymes. According to the results of the *in vivo* targeting experiments, this presequence plays a role in targeting the proteins to peroxisomes. In fact, the first 11 amino acids of the thiolase 1 prepiece constitute or contain a novel PTS which could direct bacterial chloramphenicol acetyltransferase (CAT), a normally cytosolic protein, to peroxisomes.

Studying peroxisomal thiolase has led to a number of interesting future projects. The significance of the existence of two peroxisomal 3-ketoacyl-CoA thiolases could be investigated. It will be of interest to determine if these two homologous proteins possess different characteristics such as different substrate specificities or different kinetic properties. It is intriguing that the expression of the genes encoding the two rat peroxisomal thiolases are differentially regulated. This observation has initiated ongoing investigations into the induction of expression of genes encoding peroxisomal proteins by clofibrate. Using the two peroxisomal thiolase genes as a model system, deletion analysis of the 5'-flanking regions should help to elucidate potential clofibrate-responsive elements, a step towards understanding the mechanism of action of this drug. There also exist inducible and non-inducible forms of peroxisomal fatty acyl-CoA oxidase (Schepers *et al*, 1990). It will certainly be of interest to determine why two forms of these ß-oxidation enzymes exist and whether there are also multiple

forms of the bifunctional enzyme, hydratase dehydrogenase.

Future studies should also focus on further characterization of the novel PTS of thiolase and on elucidation of the structures and functions of the components involved in each step of the transport pathway. Some questions can be addressed using a heterologous *in vivo* approach, as we have done here; however, a complete understanding of the signals and processes involved will require a reliable *in vitro* import system. The system developed by Gould *et al* (1987; 1988) that we used for thiolase is a simple and elegant assay, but it is not without limitations. Firstly, one is limited to studying proteins whose antibodies do not cross-react with endogenous proteins of the host cells or to using reporter proteins that may not be entirely compatible with the import machinery. Secondly, this assay is only qualitative giving no indication of efficiency of the import process. It will be of interest to establish the essential features of the novel peroxisomal targeting signal, whether it is sequence-dependent or simply dependent on the distribution of certain amino acid groups (*e.g.* hydrophobic or basic). It will also be interesting to determine if the targeting signal can function at different locations in the protein (*e.g.* carboxy-terminus) or whether the signal must be located near the amino-terminus for proper recognition and function. It is noteworthy that 10 amino acids precede the targeting signal in thiolase 2, which

indicates that it can function at an internal location.

It appears that thiolase uses a unique import pathway independent of the pathway that recognizes the tripeptide PTS. This point could be further confirmed by illustrating that a peptide containing the tripeptide SKL does not effect the uptake of the thiolase precursor. The thiolase prepiece could be used as a probe to identify cellular components of the peroxisomal import machinery [*e.g.* membrane-bound receptor(s) or cytosolic component(s)] that specifically interact with it. It will also be of interest to determine if the import of the thiolase precursor is coupled to proteolytic processing. It should be noted that in CV-1 cells the precursor is not cleaved upon import as determined by immunoblots of transfected cells (Dr. B. Swinkles and Dr. S. Subramani, personal communication). This result may be an indication that the two processes are not tightly coupled, and perhaps removal of the prepiece is necessary for a different purpose such as proper folding and/or functioning of the protein.

## CONCLUSIONS

Our current understanding of peroxisome biogenesis encompasses three central precepts:

1) peroxisomal proteins are encoded by nuclear genes, translated on free polysomes and posttranslationally imported into existing peroxisomes,

2) peroxisomal proteins are synthesized without precursor extensions or other posttranslational modifications,

3) new peroxisomes arise from fission of existing peroxisomes.

The peroxisomal 50 kDa IMP and rat liver peroxisomal thiolase are exceptions to what is generally accepted to occur in peroxisome biogenesis. Preliminary results demonstrating that a 50 kDa IMP is located in both peroxisomes and ER and appears to be preferentially synthesized on membrane-bound polysomes challenges the first precept of peroxisome biogenesis. Rat liver peroxisomal thiolase violates the second precept of peroxisome biogenesis because it is synthesized as a larger precursor. This brief conclusion will reiterate the contributions made by the investigations described in this thesis to our understanding of peroxisome biogenesis.

Investigations of the peroxisomal IMPs of rat liver have shown that there are homologous proteins located in

different subcellular locations (the 15, 36 and 50 kDa IMPs). This finding raises the question as to how the cell directs homologous proteins to different cellular locations. It is not yet known whether the homologous proteins are identical and whether they are encoded by one or more genes. The result that the 50 kDa IMP is co-localized to peroxisomes and ER and is preferentially synthesized on membrane-bound polysomes is a unique situation. The possibility exists that there are two isoforms of the 50 kDa IMP, one synthesized on free polysomes and posttranslationally inserted into the peroxisomal membrane and the other synthesized on membrane-bound polysomes and co-translationally inserted into the ER membrane. An alternative possibility is that there is only one isoform of the protein that is synthesized on membrane-bound polysomes and is subsequently transferred to peroxisomes. This second scenario suggests that the ER plays a role in peroxisome biogenesis, an idea that was generally abandoned years ago.

Recent evidence showing that the ER and peroxisomes cooperate in many metabolic functions makes it possible that the 50 kDa IMP participates in the functional cooperation between these organelles. For example, it may participate in a shuttle system between these two organelles. However, the 50 kDa IMP appears to be more abundant in the peroxisomal membrane as compared to the ER membrane, even though a large proportion of it is synthesized on membrane-bound polysomes. These results may imply that the ER is involved in its

biosynthesis. Although the results are too preliminary to draw any definitive conclusions, this situation may lead to a reevaluation of the first rule of peroxisome biogenesis.

Rat liver peroxisomal thiolase violates the second precept of peroxisome biogenesis, because it is synthesized as a larger precursor. The results indicate that the presequence of thiolase contains information for its targeting to peroxisomes. This presequence represents a novel peroxisomal targeting sequence (PTS); however, the essential features of this PTS remain to be elucidated. Why thiolase possesses this unique PTS is unknown, but it is clear that more than one mechanism of targeting to peroxisomes exists.

In conclusion, although generalization of a mechanism is a good way to formulate working models, there is something to be learned from studying exceptions to the rules.

## APPENDIX A

### A.1 Selection and Preliminary Characterization of a Partial cDNA Encoding the 50 kDa IMP

To facilitate future studies of the properties and biogenesis of the 50 kDa IMP, cloning of the cDNA encoding this IMP was undertaken. This section of work is as yet incomplete and is therefore included as an appendix. A partial cDNA was selected and characterized; however, the identity of this cDNA has not been conclusively determined. The results of the preliminary experiments are summarized in this section.

A rat liver cDNA λgt11 expression library was screened using monospecific affinity-purified anti-50 kDa IMP antibodies (characterized in the Results and Discussion, section 3.3.2, Fig. 3.3.3). Using the double antibody technique of Young and Davis (1983), only one positive recombinant was identified of the approximately 200,000-300,000 plaques screened. The identity of this recombinant was determined by preparing a bacterial lysate containing the ß-galactosidase fusion protein encoded by the recombinant phage, followed by immunoblotting with either anti-peroxisomal IMP serum or anti-ß-galactosidase serum. A bacterial lysate containing the recombinant antigen was prepared by expressing the λgt11 recombinant as a lysosgen in a high frequency lysogenic host (*E. coli* Y1089). During lysogenic growth,

transcription of the viral late genes (responsible for coat protein production and packaging) is repressed, and production of the ß-galactosidase fusion protein can be induced by IPTG without subsequent formation of progeny bacteriophage particles and cell lysis (Huynh *et al*, 1985). After a period of sufficient growth and expression, the cells were harvested and lysed. Both the supernatant and pellet fractions (containing bacterial membranes) were tested for the presence of a ß-galactosidase fusion protein which reacted with the anti-peroxisomal IMP serum. The immunoblot illustrated in Fig. A.1 shows that the anti-peroxisomal IMP serum reacted with a ß-galactosidase fusion protein found predominantly in the membrane fraction of *E. coli* (Fig. A.1, panel B, lane 4). This result indicated that the fusion protein was sufficiently hydrophobic to associate with the membrane fraction, suggesting that the cDNA may encode a membrane protein. Although encouraging, this result indicated only that the product of the cDNA reacted with a component of the antiserum. To obtain further proof that this recombinant encoded the 50 kDa IMP, hybridization-selection-translation experiments were performed (Parnes *et al*, 1981; Rachubinski *et al*, 1985). In these experiments, the cDNA recombinant was used to select a specific mRNA by hybridization, which was subsequently translated. The translation product was analyzed by SDS-PAGE and immunoprecipitation. Hybridization-selection-translation

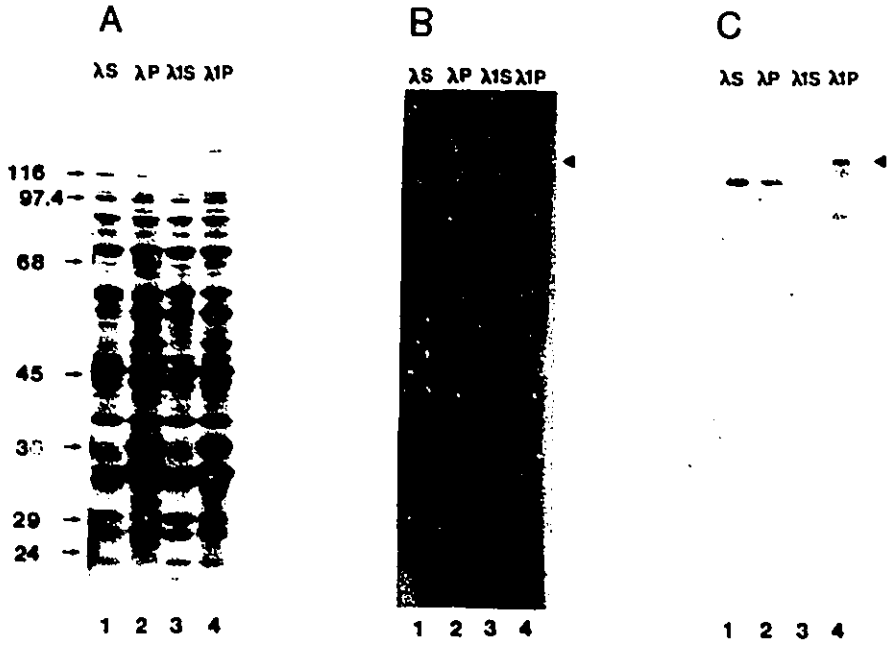



Fig. A.1. Immunoblot analyses of bacterial lysates containing the ß-galactosidase fusion protein with anti-peroxisomal IMP serum and with anti-ß-galactosidase serum. Panel A, SDS-polyacrylamide gel stained with Coomassie Brilliant Blue (20 μg protein per lane); Panel B, immunoblot treated with anti-peroxisomal IMP serum; Panel C, immunoblot treated with anti-ß-galactosidase serum. Lane 1, supernatant fraction of bacterial lysate from bacteria infected with λgt11 (λS); lane 2, pellet fraction of bacterial lysate from bacteria infected with λgt11 (λP); lane 3, supernatant fraction of bacterial lysate from bacteria infected with the recombinant phage λ50-1 (λ1S); lane 4, pellet fraction of bacterial lysate from bacteria infected with the recombinant phage λ50-1 (λ1P). Molecular mass protein standards (in kDa) are indicated at the left. ß-galactosidase is at 116 kDa, and the ß-galactosidase fusion protein is indicated by the arrowheads.

experiments were attempted twice, without success. Unequivocal evidence that the cDNA recombinant encodes the 50 kDa IMP necessitates sequencing part of the 50 kDa IMP.

DNA was isolated from the recombinant phage and digested with *Eco*RI to yield two cDNA fragments of 1.1 kbp and 0.3 kbp. Subsequently, λ DNA was partially digested with *Eco*RI (such that a proportion of cDNA fragments were liberated from the λ DNA without digesting the internal *Eco*RI site), and the resultant 1.4 kbp fragment was subcloned into the *Eco*RI site of the plasmid vector pUC118 (Fig. A.2). This recombinant plasmid is referred to as pUC50-1.

A set of nested deletion fragments was created by the procedure of Henikoff (1984). The plasmid was digested with *Pst*I and *Bam*HI and the cDNA insert was progessively digested from the *Bam*HI site using exonuclease III. The DNA ends were polished with S1 nuclease and Klenow fragment to create blunt-ends which were ligated to one another. The pUC/M13 universal primer was used to prime double-stranded dideoxynucleotide sequencing (Zhang *et al*, 1988) of the nested fragments. DNA sequence was generated across the entire length of the cDNA insert using only one strand of the cDNA as the template. A partial restriction map and sequencing strategy are shown in Fig. A.3.

The nucleotide sequence and deduced amino acid sequence of the partial cDNA recombinant encoding the 50 kDa IMP is shown in Fig. A.4 (not including the shaded region).

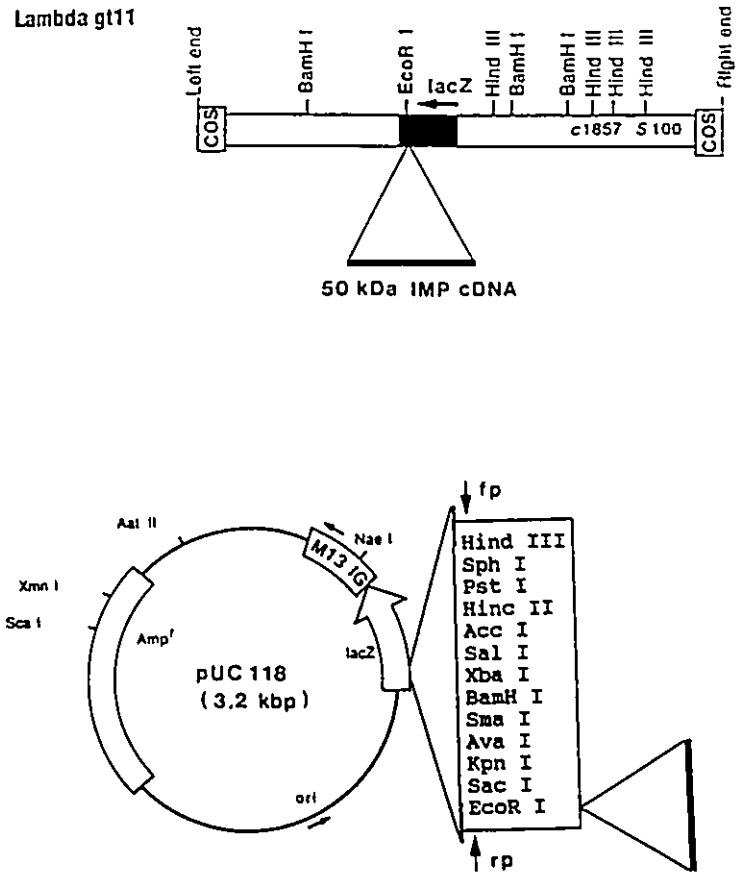



Fig. A.2. Schematic representation of the λgt11 and pUC118 vectors employed in cloning the putative rat liver peroxisomal 50 kDa IMP cDNA. A partial restriction map of λgt11 is illustrated at the top. The *LacZ* gene is darkened, and the direction of transcription is indicated by the arrow. The unique *Eco*RI site is indicated, as well as the temperature-sensitive cI repressor (c1857), the amber mutation (S100) and the cohesive ends (COS). A partial restriction map of pUC118 is shown at the bottom. The *LacZ* gene and its direction of transcription, as well as the β-lactamase coding region ($Amp^r$) are indicated. The M13 fragment used to create pUC118 from pUC18 is shown. The arrow above this fragment indicates the orientation of the phage origin of replication. The direction of the bacterial origin of replication is indicated by the arrow (*ori*). The restriction endonuclease sites present in the multiple cloning site appear in the boxed region. The binding sites of the pUC/M13 forward sequencing primer (fp) and the pUC/M13 reverse sequencing primer (rp) are shown. The 50 kDa IMP cDNA is represented by the thick line, and its site of insertion into either vector is indicated. These figures were adapted from the Promega catalogue.

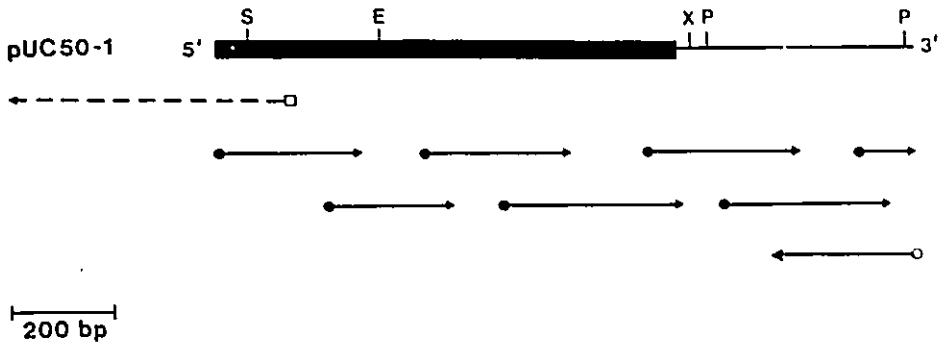



Fig. A.3. Partial restriction map and sequencing strategy for the putative cDNA encoding rat liver peroxisomal 50 kDa IMP. The partial cDNA recombinant is shown at the top with the open reading frame indicated by a thick line. E, *Eco*RI; P, *Pvu*II; S, *Sty*I; X, *Xba*I. pUC50-1 was the only recombinant immunoselected from the λgt11 rat liver library, constructed by subcloning the cDNA insert into the plasmid vector pUC118. Direction and extent of sequence determination are indicated by arrows. The solid circles represent the starting points of dideoxy chain-termination sequencing of nested deletion fragments that were created with exonuclease III using pUC50-1. The arrow with the open circle represents sequence derived from the opposite strand of DNA using the M13/pUC reverse sequencing primer. The oligo used to direct RACE primer extension is shown by an open box. The direction and extent of primer-extended cDNA are indicated by the dashed arrow.

The partial recombinant is 1,363 bp in length, lacks the 5'-end of the cDNA and contains an open reading frame of 891 bp encoding 297 amino acids (predicted $M_r$ of 33,314). The nucleotide sequence contains a typical polyadenylation sequence (AATAAA) 14 nucleotides from the 3'-end. The deduced amino acid sequence is composed of 34.9% hydrophobic amino acids, and the recombinant fusion protein is sufficiently hydrophobic to associate with the bacterial membrane fraction (Fig. A.1, Panel B, lane 4). The algorithms of Hopp and Woods (1981) and Kyte and Doolittle (1982) were used to predict the hydrophobic and hydrophilic regions of the putative protein. Both of these methods involve assigning each amino acid a numerical value (hydrophobicity/hydrophilicity value) and then progressively averaging these values along the polypeptide chain. The hydrophobicity/hydrophilicity values are based on structural and chemical properties of the amino acids, and adjustments are made to maximize how the predicted data fit well characterized proteins. There are some differences between the two algorithms in the hydrophobicity/hydrophilicity values for some amino acids, due to different choices of experimental observations derived from the literature used in each analysis. Plots of the results of these analyses on the deduced amino acid sequence of the 50 kDa IMP are shown in Fig. A.5. There are a few hydrophobic regions of sufficient length to span a membrane which may represent potential membrane spanning domains. A segment from

Fig. A.4. Nucleotide sequence and deduced amino acid sequence of the partial cDNA recombinant for the 50 kDa IMP. Nucleotides are numbered designating the first nucleotide of the partial cDNA recombinant pUC50-1 as +49. The TGA stop codon is marked with asterisks. The sequence underlined with a triple line represents a typical polyadenylation sequence. Primer extension of mRNA was conducted using a primer complementary to the sequence underlined with a thin line (AB792). The doubly-underlined sequence is the region to which an oligodeoxyribonucleotide probe was synthesized for PCR amplification of the newly synthesized cDNA in the RACE protocol (AB496). The sequence outlined by the box is the region to which a complementary oligodeoxyribonucleotide was synthesized for colony hybridization and for Southern blot, northern blot and slot blot analyses (AB497). The shaded region represents additional sequence which was generated by primer extension. The tripeptides underlined by thick lines represent possible glycosylation signals.

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                    TTA AGT GAG GAA GAG AGC GTT GGT TCT CCC GAA ACA GAA GAG ATG GCT   48
                    Leu Ser Glu Glu Glu Ser Val Gly Ser Pro Glu Thr Glu Glu Met Ala   16

TTC TTC AGC ACC CAG CCT CCA TAC ATG AAC CCA GTC ATC CCC TTT ACT GGA ATA ATC CAA  108
Phe Phe Ser Thr Gln Pro Pro Tyr Met Asn Pro Val Ile Pro Phe Thr Gly Ile Ile Gln   36

GGA GGG TTG CAG AAC GGA CTT CAG ATC ACC CTC CAG GGG ACC GTC CAC CCT TTT CCA AAT  168
Gly Gly Leu Gln Asn Gly Leu Gln Ile Thr Leu Gln Gly Thr Val His Pro Phe Pro Asn   56

AGG ATT GCG GTG AAC TTT CAG ACT GGC TTC AGT GGA AAT GAC ATT GCC TTC CAC TTC AAT  228
Arg Ile Ala Val Asn Phe Gln Thr Gly Phe Ser Gly Asn Asp Ile Ala Phe His Phe Asn   76

CCC CGG TTT GAG GAA GGA GGA TAT GTG GTT TGC AAC ACA AAG CAG AAT GGA AAG TGG GGG  288
Pro Arg Phe Glu Glu Gly Gly Tyr Val Val Cys Asn Thr Lys Gln Asn Gly Lys Trp Gly   96

CCT GAG GAG AGG AAG ATG CAG ATG CCC TTC CAG AAG GGG ATG CCC TTT GAG CTT TGC TTC  348
Pro Glu Glu Arg Lys Met Gln Met Pro Phe Gln Lys Gly Met Pro Phe Glu Leu Cys Phe  116

CTG GTA CAG AGG TCG GAA TTC AAG GTG ATG GTG AAC AAG AAC TTC TTT GTA CAG TAC TCA  408
Leu Val Gln Arg Ser Glu Phe Lys Val Met Val Asn Lys Asn Phe Phe Val Gln Tyr Ser  136

CAC CGC GTG CCC TAC CAC CTC GTG GAC ACC ATT TCG GTC TCG GGA TGC TTG CAC CTG TCC  468
His Arg Val Pro Tyr His Leu Val Asp Thr Ile Ser Val Ser Gly Cys Leu His Leu Ser  156

TTC ATC AAC TTC CAG ACT CAG GGC TTT CAG CCT GCC CAC CAG GCA CCC GTG GCT CAA ACT  528
Phe Ile Asn Phe Gln Thr Gln Gly Phe Gln Pro Ala His Gln Ala Pro Val Ala Gln Thr  176

ATC ATC CAC ACA GTT CAC AGC ATC CCT GGA CAG ATG CTC TCT ACT CCT GGA ATC CCT CCT  588
Ile Ile His Thr Val His Ser Ile Pro Gly Gln Met Leu Ser Thr Pro Gly Ile Pro Pro  196

ATG GCA TAC CCC ACC CCA GCC TAT ACT ATA CCT TTC TTC ACC AGC ATC CCA AAT GGG TTT  648
Met Ala Tyr Pro Thr Pro Ala Tyr Thr Ile Pro Phe Phe Thr Ser Ile Pro Asn Gly Phe  216

TAC CCA TCC AAG TCC ATC AAC ATA TCA GGC GTG GTC TTG CCA GAT GCT AAG AGG TTC CAT  708
Tyr Pro Ser Lys Ser Ile Asn Ile Ser Gly Val Val Leu Pro Asp Ala Lys Arg Phe His  236

ATC AAC CTT CGC TGT GGG GGT GAC ATT GCT TTC CAC CTG AAC CCC CGT TTC AAT GAG AAG  768
Ile Asn Leu Arg Cys Gly Gly Asp Ile Ala Phe His Leu Asn Pro Arg Phe Asn Glu Lys  256

GTT GTG GTC CGA AAC ACT CAG ATC AAC AAC TCC TGG GGG CCC GAG GAG CGA AGC TGC CTG  828
Val Val Val Arg Asn Thr Gln Ile Asn Asn Ser Trp Gly Pro Glu Glu Arg Ser Cys Leu  276

GGA GAA TGC CCT TCA ATC GTG GCC AGA GTT TCT CAG TGT GGA TCT TAT GTG AAG GTC ACT  888
Gly Glu Cys Pro Ser Ile Val Ala Arg Val Ser Gln Cys Gly Ser Tyr Val Lys Val Thr  296

GCT TCA AGG TGG CCG TGG ATG GTC AGC ATA TTT GTG AAT ATT ACC ACC GCC TGA AGAACTT  949
Ala Ser Arg Trp Pro Trp Met Val Ser Ile Phe Val Asn Ile Thr Thr Ala ***          313

GCCGGATATCAACACTCTAGAGGTGGCCGGTGATATCCAGCTGACACACGTGCAGACCTAGGAAGGTCTCTGGCTTAGG 1028

GATGAAGGCTGAGGAACCCTACCTGAGTCTTGTCACCTCCTCCCTGTCTCAGCCCTGCCTCCCCAAATCCTGTCATCAA 1107

AGAGAGCCTCATTGGCAGGAGTTCCAGGAAGGTGGCATTCCCAATTCACACCCTCCACAAAGGGGGAGTCCTGGGCTAT 1186

GGGACACATGGCTGTGAGCCCACAGTGTCAGCCATTGCTCCCTAGCTAGTCATCTTCTGAGGGAAGTGACCTCCCTGGG 1265

TTTGCCCCTTTCTCTGACCTTTCCCTTCACCCCTCCAGGAGGGCCACCTTGATGTCATCCCATTGGCCTCCAGCTGACC 1344

CAGAATGTCCACATTACCTTTTCCCCAATCTTTCCCAATGCCCATAAAATAAAGAATATCAACGCTC              1411
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amino acid 16 to amino acid 38 is hydrophobic by Hopp and Woods (1981) analysis, and 15 of these (amino acids 22-36) are also hydrophobic by Kyte and Doolittle (1982) analysis. Amino acids 144 to 165 are hydrophobic by Hopp and Woods (1981), and 18 amino acids (amino acids 142 to 159) form a potential membrane spanning segment by Kyte and Doolittle (1982) analysis. In addition, the last 17 amino acids and 12 amino acids of the deduced sequence are also hydrophobic by Hopp and Woods (1981) and Kyte and Doolittle (1982) analysis, respectively.

The deduced amino acid sequence does not contain a peroxisomal tripeptide targeting sequence (SKL) or an acceptable variation thereof. This is not unexpected, as it has been suggested that the peroxisomal membrane proteins may use a type of PTS different from that of soluble peroxisomal proteins with a carboxy-terminal SKL tripeptide (Santos *et al*, 1988a; 1988b; Gould *et al*, 1990b).

It is interesting that the deduced amino acid sequence also includes three putative glycosylation sites. The significance of this observation awaits confirmation of the absolute identity of the recombinant and further analysis of the purified protein. It would be of interest to determine if either the peroxisomal or ER form of the 50 kDa IMP is glycosylated. The peroxisomal and ER forms of the 50 kDa IMP comigrate in SDS-PAGE, indicating that they are of similar molecular weight. Therefore if glycosylated, they are

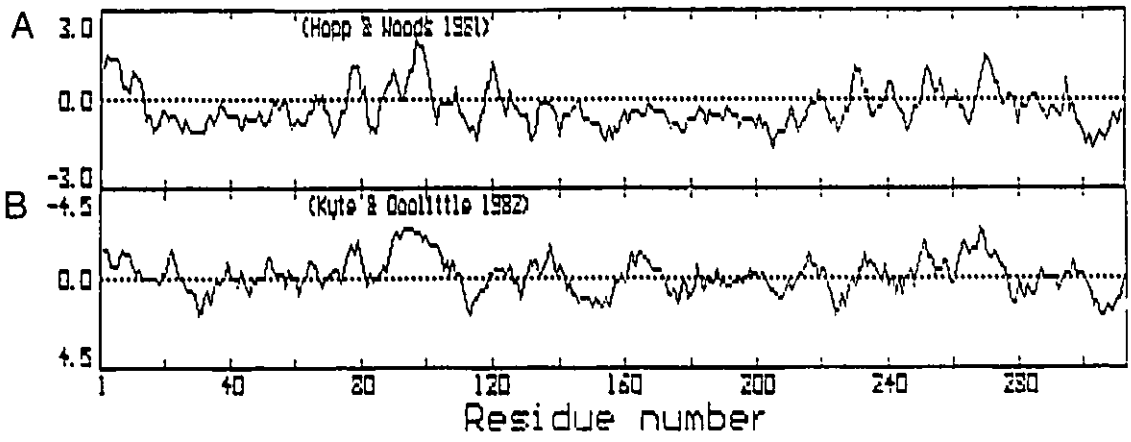


Fig. A.5. Hydrophobicity/hydrophilicity profiles determined for the deduced amino acid sequence of the 50 kDa IMP cDNA. Panel A, shows the hydrophobicity profile determined by the algorithm of Hopp and Woods (1981) using a window size of 6. Panel B, shows the hydrophobicity profile generated by the algorithm of Kyte and Doolittle (1982) using a window size of 9. The horizontal axis denotes sequence position. The vertical axis represents the range of hydrophobicity/hydrophilicity values. In Panel A the negative numbers represent hydrophobic values, while in Panel B, the positive numbers represent hydrophobic values.

glycosylated to similar extents. If they are both glycosylated with sugars characteristic of those added in the secretory pathway, this argues for the peroxisomal form entering the ER prior to localization in the peroxisomal membrane. However, unless the carbohydrate moieties are of low molecular weight or there is substantial processing of the polypeptides, we suspect glycosylation does not take place, as the *in vitro*-synthesized protein comigrates with the mature protein in SDS-PAGE. In addition, there is no definitive evidence for the glycosylation of peroxisomal proteins (Lazarow and Fujiki, 1985).

## A.2 Analysis of the mRNA Encoding the 50 kDa IMP

Poly(A)$^+$RNA was isolated from the livers of untreated and clofibrate-treated rats and used in Northern blot analyses. The blots were probed with a $^{32}$P-labeled oligodeoxyribonucleotide specific to the coding strand of the cDNA encoding the 50 kDa IMP (Fig. A.4, probe AB497). The mRNA encoding the 50 kDa IMP was approximately 1.8 kb in size and appeared to be induced slightly upon treatment with clofibrate (Fig. A.6, panel A). This level of induction was quantitated using RNA slot blot analysis (Fig. A.6, panels B and C). Poly(A)$^+$RNA samples were applied to nitrocellulose in step-wise decreasing increments, and the blots were hybridized with the $^{32}$P-labeled oligodeoxyribonucleotide probe AB497, using hybridization and wash conditions identical to those

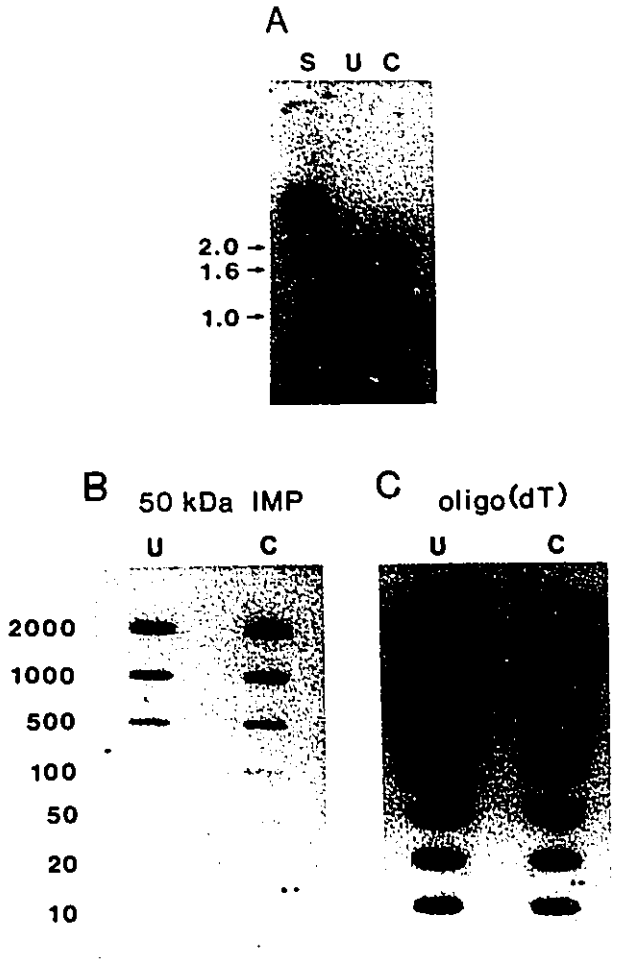


Fig. A.6. Analysis of the mRNA encoding the 50 kDa IMP. Panel A, northern blot analysis of rat liver poly(A)$^+$RNA isolated from livers of untreated (U) and clofibrate-treated (C) rats, probed with $^{32}$P-labeled oligodeoxyribonucleotides specific for the putative 50 kDa IMP mRNA (probe AB497). DNA markers are shown in lane S and the sizes (in kb) are indicated on the left margin. Panel B, slot blot analysis of liver poly(A)$^+$RNA from untreated (U) and clofibrate-treated (C) rats probed with an oligodeoxyribonucleotide specific for the putative 50 kDa IMP mRNA (AB497). The number of nanograms of poly(A)$^+$RNA loaded in each well is indicated at the left. Panel C, for quantitation, the slot blot was washed and reprobed with $^{32}$P-labeled oligo(dT)$_{18}$.

used in northern blot analysis (Fig. A.6, panel B). The blots were standardized for the amount of poly(A)$^+$RNA loaded by washing the initial probe off the blots and reprobing the blots with radiolabeled oligo$(dT)_{18}$ (Fig. A.6, panel C; Harley, 1987). The level of induction was quantitated by densitometric analysis of the resultant autoradiograms. After correction for errors resulting from unequal amounts of mRNA in adjacent lanes of the slot blot, the induction of the 50 kDa IMP mRNA was 1.99 ± 0.3-fold (n=2). This amount of induction was similar to the increase in the 50 kDa IMP seen in the peroxisomal membrane of clofibrate-treated rats (2.93 ± 1.18-fold) (Results and Discussion, section 3.4).

The size of the mRNA is sufficiently large to encode a 50 kDa protein, but the non-coding regions must be quite small. This small size of mRNA brings into question the identity of the cDNA clone, but the induction of the mRNA upon clofibrate treatment is at least suggestive that the cDNA encodes the 50 kDa IMP. It is interesting that only one species of mRNA was detected on northern blots using both the oligodeoxyribonucleotide probe (AB497) and the full-length cDNA insert as a probe (data not shown). This result may imply that both forms of the 50 kDa IMP (peroxisomal and ER) arise from a single mRNA. However, the possibility exists that the two forms of the protein arise from distinct mRNAs which do not cross hybridize.

## A.3 Primer Extension of the mRNA Encoding the 50 kDa IMP

In order to complete the 5′-end of the cDNA for the 50 kDa IMP, RACE primer extension was undertaken. The protocol used was identical to that used to complete the 5′-end of the cDNA encoding peroxisomal thiolase 2 (Bodnar and Rachubinski, 1990). Primer AB792, (complementary to the region underlined by a thin line in Fig. A.4) was used to direct primer extension from mRNA. Because the cDNA insert of the recombinant was 1.4 kbp and the mRNA was 1.8 kbp we expected to extend the cDNA approximately 400 bp from the 5′-end of the partial recombinant or approximately 550 bp from the primer used in the reverse transcriptase reaction. The products of the reverse transcriptase reaction were analyzed on a denaturing 4% polyacrylamide gel which resolved two major bands, one of approximately 325 nucleotides and the other of about 550 nucleotides (Fig. A.7, panel A). The shorter product may represent a prematurely terminated cDNA, perhaps arising from a region in the mRNA with a propensity to form secondary structure that causes the reverse transcriptase to dissociate from the template. To address this issue, the reaction was repeated at higher temperatures (50°C and 65°C) using Taq polymerase, which has been shown to possess reverse transcriptase activity (Jones and Foulkes, 1989). This reaction was performed using both primer AB792 and primer AB496 (located about 50 bp upstream of AB792, see Fig. A.4) and, as expected, both primers produced primarily a fragment

of approximately 500 bp in length (Fig. A.7, panel B). Together these results indicated that there remained approximately 400 nucleotides of sequence to be determined at the 5'-end of the cDNA and that a region may exist in the mRNA which forms a secondary structure at lower temperatures. This first strand cDNA was tailed at the 5'-end using dATP and terminal deoxynucleotidyl transferase and second strand cDNA was synthesized using the oligo$(dT)_{17}$-adaptor (AB703). The resultant cDNA was PCR amplified using primers AB496 (see Fig. A.4) and the adaptor primer (AB705). The PCR reaction products were run on a 4% agarose gel (Fig. A.7, panel C) and yielded only a band at approximately 200 bp and a smear of DNA. This material was transferred to nitrocellulose and hybridized with probe AB497 to identify any products specific to the 50 kDa IMP cDNA (Fig. A.7, panel D). Southern blot analysis identified two specific DNA fragments of about 200 bp and 350 bp arising from the reaction using reverse transcriptase to synthesize the first-strand cDNA, and a product of 200 bp resulting from the reaction using Taq polymerase to synthesize the first-strand cDNA. The 350 bp fragment probably corresponds to the band at this molecular weight from the first-strand cDNA reaction products (Fig. A.7, panel A); however, the origin of the 200 bp fragment is unclear. There was no predominant band at this molecular weight present among the first-strand cDNA reaction products. In an attempt to amplify the larger DNA fragments, the PCR

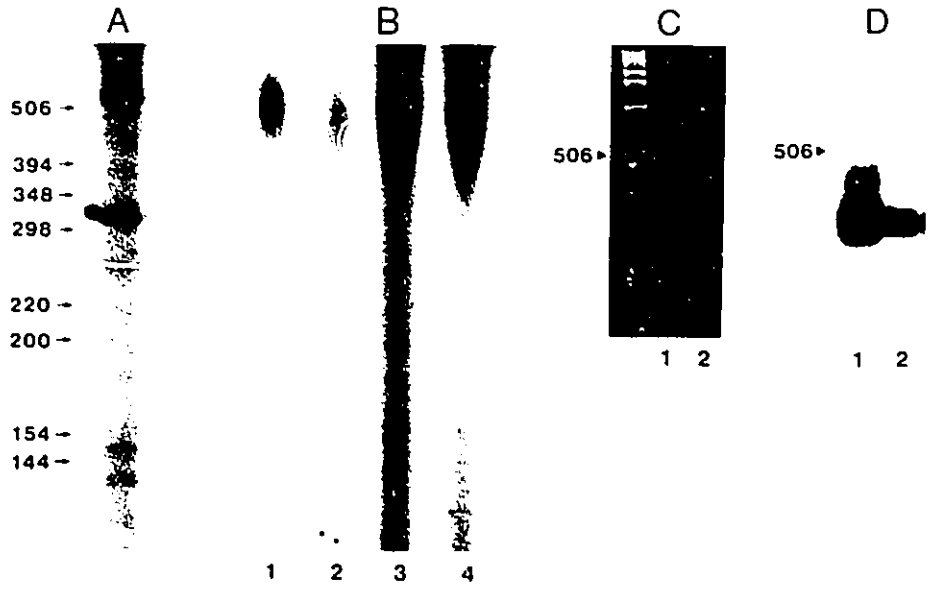


Fig. A.7. Products of the RACE primer extension procedure for the 50 kDa IMP cDNA. Panel A, autoradiogram of the $^{32}$P-labeled first-strand of cDNA synthesized by primer extension using the primer AB792 and reverse transcriptase resolved on a denaturing 4% polyacrylamide gel; Panel B, autoradiogram of the $^{32}$P-labeled first-strand cDNAs synthesized using Taq polymerase; lane 1, primer AB792 at 50°C; lane 2, primer AB792 at 65°C; lane 3, primer AB496 at 50°C; lane 4, primer AB496 at 65°C; Panel C, The PCR reaction products were electrophoresed in a 4% agarose gel; lane 1, the reaction products derived from the first-strand cDNA synthesized using reverse transcriptase and primer AB792; lane 2, the reaction products derived from the first-strand cDNA synthesized with Taq polymerase using primer AB792 at 50°C; Panel D, corresponding Southern blot of the PCR reaction products probed with oligodeoxyribonucleotide AB497. Molecular size standards (in nucleotides) are indicated at the left of Panel A. The molecular size standards shown in Panels B and C are identical to those marked in panel A (in bp), but only the 506 bp marker is labeled for reference.

reaction was repeated using several different Taq polymerase buffers and various temperatures for the steps in the PCR reaction; however, no larger fragments were amplified. The reason for this is unknown, but it may be a result of stable secondary structure in the mRNA that prevents the polymerase from further extension. However, this seems unlikely as the temperature of the PCR reaction never dropped below 50°C.

The ends of the cDNA products were polished using Klenow fragment, digested with *Xho*I (which recognizes a site in the adaptor at the very 5'-end of the cDNA) and subcloned into the plasmid pGEM-7Zf(+) (previously digested with *Sma*I and *Xho*I). The clones containing cDNA inserts were selected as white colonies on LB agar plates containing 100 μg/ml ampicillin and X-gal. Colonies containing plasmids harbouring the insert for the 50 kDa IMP cDNA were selected by colony hybridization with the probe AB497. Plasmids were isolated from these colonies, and the sizes of the cDNA inserts were determined by restriction endonuclease analysis followed by agarose gel electrophoresis. This analysis revealed that all the clones selected had small cDNA inserts (approximately 200 bp). Sequencing of some of these inserts extended the sequence of the 50 kDa IMP cDNA by only about 50 bp (Fig. A.4, shaded region). The reason for the inability to subclone larger cDNA pieces is unknown; however, it is suspected that the expression of this portion of cDNA as a β-galactosidase fusion product in the Y1090 bacteria cells may kill the cells.

Perhaps this portion of the cDNA encodes a highly hydrophobic membrane spanning domain which is inserted into the bacterial membrane, interupting life-essential processes. For this reason, the cDNA inserts were subcloned into the plasmid pSP72 (Promega Corp.), which does not contain the *LacZ* complementation system. The cDNA inserts were blunt-ended and digested with *Cla*I and *Sma*I and subcloned into pSP72 which had been similarly digested. This plasmid was introduced into *E. coli* (DH5α) cells, and colonies harbouring the cDNA insert of interest were selected with probe AB497. The cDNA inserts from 150 independent plasmids were investigated, but again all inserts were small in size (approximately 200 bp). The reasons for the inability to subclone larger inserts is unknown.

In an attempt to generate more sequence data the larger PCR reaction products were purified by electroelution and the isolated fragments were used for direct DNA sequencing (Fernandez-Rachubinski *et al*, 1990). Unfortunately, this experiment was unsuccessful.

## A.4 Comparison of the Deduced Amino Acid Sequence of the 50 kDa IMP cDNA with Sequence Data Banks

In order to further characterize the partial cDNA, a search of its deduced amino acid sequence with the Genetic Sequence Data Bank (GenBank) and the National Biomedical Research Foundation (NBRF) Protein Data Bank was performed.

The protein encoded by the 50 kDa IMP cDNA shows regions of homology to a rat IgE-binding protein as well as a class of soluble mammalian lectins.

The IgE-binding proteins are a group of cell-surface receptors of mast cells, basophils, lymphocytes and other cell types which mediate various IgE related cellular responses (Liu *et al*, 1985). There are two regions of identity to a rat 31 kDa IgE-binding protein, including amino acids 37 to 151 which exhibit 48.7% identity and amino acids 205 to 273 which show 49.3% identity. This striking similarity raises the possibility that this recombinant has been selected because of its similarity to an immunoglobulin-binding protein. The reaction of the ß-galactosidase fusion protein with pre-immune serum was not investigated but would clarify this possibility.

The protein encoded by the 50 kDa IMP cDNA also has sequence identity with various lectins. Amino acids 66 to 116 of our protein show 40-50% identity to a region of ß-galactoside-binding lectins from various mammalian species (Ohyama *et al*, 1986; Southan *et al*, 1987; Paroutaud *et al*, 1987; Hirabayashi *et al*, 1987, Clerch *et al*, 1988). In addition, amino acids 37 to 151 show 47.9% and amino acids 185 to 273 exhibit 45.4% identity to two regions of the mouse galactose-specific lectin (Jia and Wang, 1988). These results suggest that the protein encoded by the recombinant may be a polypeptide with carbohydrate-binding activity. However, the *in vivo* function of this class of mammalian lectins is not yet

clear, and the sugar-binding site has not been confirmed. Therefore, it is difficult to evaluate the significance of this homology (Southan *et al*, 1987).

Recently, two cDNAs encoding rat liver peroxisomal IMPs have been cloned, one for a 70 kDa IMP and a second for a 35 kDa IMP. The 70 kDa IMP has similarity to members of the ATP-binding protein family, which includes several proteins involved in bacterial transport systems and the protein that confers multi-drug resistance to mammalian tumor cells (P-glycoprotein) (Kamijo *et al*, 1990). It is suspected that this protein is involved in active transport across the peroxisomal membrane (Kamijo *et al*, 1990). The 35 kDa IMP restores the biogenesis of peroxisomes in a peroxisome defective mutant of CHO cells and it has been speculated that it is a component of a putative signal receptor or the transmembrane import machinery of peroxisomes (Tsukamoto *et al*, 1991). Upon comparison, there were no obvious sequence homologies between either of these sequences and the deduced amino acid sequence for the 50 kDa IMP.

## A.5 Summary

Although a partial cDNA recombinant has been selected for the 50 kDa IMP, the preliminary results do not verify its identity. The recombinant was selected with affinity purified anti-50 kDa IMP antibodies and was shown to react with a component of the anti-peroxisomal IMP serum. Confirmation

that the selected recombinant encodes the 50 kDa IMP awaits hybridization-selection-translation experiments followed by characterization of the translation product or ideally, sequencing of part of the protein. Upon confirmation of its identity, it should be determined if this recombinant encodes the peroxisomal form or ER form of the 50 kDa IMP or whether there is only one form of this protein. Experiments suggested in the Results and Discussion (section 3.6) should help address these questions.

The reasons for the difficulty in completing the 5'-end of the cDNA recombinant are unknown. Perhaps a new approach should be taken to obtain this portion of the cDNA. For example, attempt to amplify by PCR the 5'-region of the cDNA directly from a cDNA library using a specific primer and a primer within the vector sequence. Alternatively, the partial recombinant could be used in an attempt to select a larger cDNA recombinant from the recently developed 5'-STRETCH™ libraries (Clontech). These libraries have been designed to provide a greater percentage of full-length cDNAs.

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## LIST OF PUBLICATIONS

### PAPERS

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### ABSTRACTS

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