TARGETING AND IMPORT OF THE PEROXISOMAL 3-KETOACYL-CoA THIOLASE OF *SACCHAROMYCES CEREVISIAE*

By

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ABSTRACT

The mechanism by which subcellular organelles are assembled is one of the fundamental problems of eukaryotic cell biology and biochemistry. Proteins synthesized in the cytosol are targeted to the appropriate membrane by signals usually encrypted in some primary sequence segment. Many peroxisomal proteins are targeted by the C-terminal tripeptides which are identical to or conserved variants of Ser-Lys-Leu. Mammalian peroxisomal 3-ketoacyl-CoA thiolases are targeted by a cleavable N-terminal presequence. In this thesis the targeting of *Saccharomyces cerevisiae* thiolase was investigated. The N-terminal 16 amino acids of *S. cerevisiae* thiolase were shown to be both necessary and sufficient for peroxisomal targeting in yeast. Unlike mammalian thiolases, the native thiolase of *S. cerevisiae* is not detectably modified by cleavage of the targeting sequence. Several amino acid residues within the targeting region that are conserved among all thiolases were altered by mutagenesis, and three critical residues were identified-- Arg4, Leu5, and Leu12. A novel approach was used to demonstrate that prior to translocation into peroxisomes, thiolase can form dimers. A targeted subunit could mediate the import of a cytosolic variant of thiolase by this means. The implications of this observation with respect to the conformation of thiolase prior to and during translocation are discussed.

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When I arrived at the Rach lab I had some difficulty understanding the jargon of molecular biology having had no previous exposure. For the first while I had to ask a lot of questions and strain the patience of everyone in the lab. But in the long run, my goal of learning the arcana of the gene jockey was realized.

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Finally I'd like to dedicate this thesis to my wife Sylvia Kraus. The many late nights and weekends that I spent in the lab that went into this work are also dedicated to her.

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AOX	acyl-CoA oxidase
AP	alkaline phosphatase
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indolylphosphate
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CEN	centromeric sequence
Ci	Curie
CIP	calf intestinal phosphatse
cm	centimeter
CoA	coenzyme A
CoASH	underivatized coenzyme A
Da	Dalton
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
DOC	deoxycholate
DTT	dithiothreitol
ECL	Enhanced Chemiluminescence™
EDTA	ethylenediaminetetraacetic acid
g	gram
g	gravity
h	hour
HRP	horseradish peroxidase
HSA	human serum albumin
IgG	immunoglobulin G
k	kilo
L	litre
LB	Luria broth
LPA	linear polyacrylamide
M	molar
m	milli
mAb	monoclonal antibody

MES	2-(N-morpholino)ethane sulfonic acid
mol	mole
M_r	molecular mass (relative)
n	nano
NBT	nitro blue tetrazolium
NC	nitrocellulose
O/N	overnight
p	pico
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
PNK	polynucleotide kinase
PrA	Protein A
PTS1	peroxisomal targeting signal type 1
PTS2	peroxisomal targeting signal type 2
RNA	ribonucleic acid
RT	room temperature
SCIM	yeast culture medium
SCT	*Saccharomyces cerevisiae* 3-ketoacyl-CoA thiolase
SDS	sodium dodecylsulfate
SSC	saline sodium citrate
TBE	Tris-borate-EDTA buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.05% w/v TWEEN 20
TE	10 mM Tris-HCl (pH 8.0), 1mM EDTA
TEMED	N,N,N',n'-tetramethylethylenediamine
TLCK	Nα-p-tosyl-L-lysine chloromethyl ketone
TPR	tetratricopeptide repeat
Tris	tris(hydroxymethyl)aminoethane
Tween 20	polyoxyethylenesorbitan monolaurate
Tween 40	polyoxyethylenesorbitan monopalmitate
TX-100	Triton X-100 (phenoxy polyethyloxy ethanol)
U	unit (enzyme activity)
UAS	upstream activating sequence
URS	upstream regulatory sequence
UV	ultraviolet
V	volt
VLCFA	very long chain fatty acid
w/v	weight per unit volume
YEPD	yeast growth medium
YNB	yeast nitrogen base
YNBD	yeast culture medium
YNO	yeast culture medium
μ	micro

1. INTRODUCTION

1.1. OVERVIEW

Eukaryotic cells harbour a complex array of intracellular membranes which allow certain metabolic reactions to be compartmentalized. Some protein components of mitochondria and chloroplasts are synthesized intracompartmentally, but the majority of proteins which make up each subcellular compartment are synthesized in the cytosol and imported across, or integrated into, the appropriate membrane(s). The specificity of this process is assured by the interaction of targeting signals usually encrypted in the amino acid sequence of the newly synthesized protein and components of the import machinery required by each organelle. The targeting signals and many components of the translocation machinery specific for mitochondria, endoplasmic reticulum, nucleus, and chloroplasts have been identified, although details of the translocation process remain to be elucidated in each case.

Comparatively little is known about the import of proteins into peroxisomes perhaps because peroxisomes were only recently identified as important subcellular compartments (de Duve and Baudhuin, 1966). The key discoveries that opened the frontiers of research in peroxisome biogenesis are as follows.

1. The observation that peroxisome matrix enzymes and membrane proteins are synthesized on free polysomes and imported into pre-existing peroxisomes weaned investigators away from earlier notions that peroxisomes were formed by budding from

the endoplasmic reticulum (Goldman and Blobel, 1978; Roa and Blobel, 1983; Fujiki *et al.*, 1984, 1985; Rachubinski *et al.*, 1984).

2. The discovery that human fibroblasts from patients with Zellweger syndrome contained peroxisomal "ghosts"– apparently normal membrane structures devoid of matrix components– lent urgency to the elucidation of matrix enzyme import pathways and the identification of genetic defects related to this and other disorders of peroxisomal assembly (Santos *et al.*, 1988).

3. The identification of the first peroxisomal targeting signal in firefly luciferase and the subsequent findings that C-terminal tripeptide signals were essential for microbody import in a broad range of eukaryotic organisms (Gould *et al.*, 1987, 1988, 1989, 1990a, 1990b; Aitchison *et al.*, 1991, 1992a) represented the discovery of a novel, surprisingly compact targeting motif.

4. The identification of an N-terminal targeting motif in rat liver peroxisomal thiolase (Swinkels *et al.*, 1991) supported the idea that at least two distinct import pathways mediated by C- and N-terminal signals are required for peroxisome assembly.

5. The development of peroxisome assembly mutants (*pas*) in *S. cerevisiae* (Erdmann *et al.*, 1989) promised rapid identification of genes encoding assembly factors and stimulated a flurry of similar approaches to the identification of genes in other less conventional yeasts.

Conspicuously absent from this list of achievements is the development of a robust *in vitro* system for the reconstitution of peroxisomal import. Reports of such systems

have appeared sporadically in the literature (Gietl and Hock, 1984; Small and Lazarow, 1987; Small *et al.*, 1988; Imanaka *et al.*, 1987; Fujiki *et al.*, 1989; Sommer *et al.*, 1990; Thieringer *et al.*, 1991; Miura *et al.*, 1992; Behari and Baker, 1993; Soto *et al.*, 1993). The extent of import, measured as resistance to externally applied protease, is always low and none of these systems appears to have lead to a sustained line of investigation. The biophysical characteristics of isolated peroxisomes do not reflect those of the *in situ* organelle (see discussion of peroxisome membrane below). Therefore, a "positive" indication of *in vitro* peroxisomal import may not signify the legitimate reconstitution of the cellular pathway. Recently reported reconstitution of peroxisomal import in permeabilized mammalian cells (Rapp *et al.*, 1993; Wendland and Subramani, 1993a, 1993b) offers similar analytical opportunities as *in vitro* import without subjecting the organelles to the rigors of isolation procedures.

The period in which the research for this thesis has been conducted has been an exciting time for peroxisome biogenesis. Genes essential for peroxisome biogenesis have been isolated in both mammalian and yeast systems, and many more will be forthcoming in the next year or two. Still, few details of the import pathways are available. This thesis and the publications arising from it (Janiak *et al.*, 1994; Glover *et al.*, 1994a, 1994b) are intended to make a contribution to this end. These experiments focus on the identification and characterization of the N-terminal peroxisomal targeting signal of *S. cerevisiae* 3-ketoacyl-CoA thiolase and the description of a novel import pathway for a cytosolic version of this protein.

1.2. OCCURRENCE OF PEROXISOMES

Microbodies, a general term for peroxisomes and related organelles, are found in nearly all species of eukaryotic organisms. Some primitive protists (for example *Trichomonas vaginalis*) contain organelles known as hydrogenosomes which are no longer considered to be part of the microbody family (Johnson *et al.*, 1993). The term peroxisome was coined by de Duve (de Duve and Baudhuin, 1966) to reflect the metabolic specialization of the organelle in oxidative metabolism of a range of substrates with the formation of hydrogen peroxide as a by-product. Glyoxysomes which harbour the enzymes of the glyoxylate pathway in plants, and glycosomes which sequester enzymes of the glycolytic pathway in the trypanosomatidae, complete the microbody family of organelles. Beyond the structural similarities among microbodies with diverse metabolic functions, evidence to suggest that all types of microbodies are elaborations of a common ancestral organelle derives from the recent observation that at least one peroxisomal targeting signal (SKL-COOH) is recognized by all three types of microbodies (Gould *et al.*, 1990b; Blattner *et al.*, 1993).

In *S. cerevisiae*, peroxisomes were first detected in glucose-grown cells and were characterized by electron microscopy as small, non-mitochondrial bodies bounded by a single membrane and containing particulate (organellar) catalase (Avers and Federman, 1968). Several other yeasts were, however, more amenable to the study of peroxisome biology, because the organelles could be induced to proliferate strongly by growth on specific carbon sources. *Candida tropicalis* grown on alkanes and *Hansenula polymorpha*

grown on methanol are notable examples of non-conventional yeasts with strongly inducible peroxisome proliferation. It was not until 1987 that peroxisome proliferation in *S. cerevisiae* was found to be responsive to growth on oleic acid (Veenhuis *et al.*, 1987). The strong molecular genetic systems available in this yeast make it a prime target for current research in the field of peroxisome biogenesis (for reviews see Kunau and Hartig, 1992; Höhfeld *et al.*, 1992; Kunau *et al.*, 1993).

1.3. GENERAL STRUCTURAL PROPERTIES OF PEROXISOMES

Peroxisomes viewed in thin sections usually appear to be roughly spherical organelles bound by a single lipid bilayer membrane surrounding a relatively dense matrix. In some cell types the peroxisome appears to be reticular in form as determined by serial sectioning and 3-dimensional reconstruction (Yamamoto and Fahimi, 1987; Gorgas, 1987). The morphology of peroxisomes may be constantly shifting and the observation of reticular structures may represent intermediates captured in the process of division and growth.

Unlike chloroplasts or mitochondria, peroxisomes contain no DNA (Kamiryo *et al.*, 1982). All protein components of the organelle must therefore be encoded by nuclear genes, translated in the cytosol and imported into the peroxisomal matrix or integrated into the peroxisomal membrane.

Treatments designed to disrupt the peroxisomal membrane followed by centrifugation indicate that some matrix proteins are preferentially retained in particulate

form (Hayashi *et al.*, 1981; Alexson *et al.*, 1985; Poole and Crane, 1992). This may be taken as evidence that the peroxisome matrix retains some level of intraorganellar protein organization consisting of protein-protein interactions which are independent of the intact membrane.

In *S. cerevisiae* the peroxisomes have a density of about 1.23 $g \cdot cm^{-3}$ (Lewin *et al.*, 1990) as judged by density gradient centrifugation, reflecting the high protein content vs. other organelles such as the mitochondria (1.17 $g \cdot cm^{-3}$; Nuttley *et al.* 1990). Glucose-grown yeast have as few as a single small peroxisome (Avers and Federman, 1968; Thieringer *et al.*, 1991) whereas following induction on oleic acid the peroxisomes grow in size and proliferate and can account for 8 to 11% of the total cytoplasmic volume (Höhfeld *et al.*, 1992).

1.4. STRUCTURE AND FUNCTION OF PEROXISOME MEMBRANES

The peroxisomal membrane of *S. cerevisiae* contains only three major polypeptides of 32, 31, and 24 kDa as judged by staining of carbonate extracted membranes in SDS-polyacrylamide gels (McCammon *et al.*, 1990; Goodman *et al.*,1992). The *PAS3* gene encodes a protein required for peroxisome assembly in *S. cerevisiae,* which appears to be a 48 kDa integral peroxisomal membrane protein with a large cytoplasmic C-terminal domain (Höhfeld *et al.*,1991). The inability to detect this protein in SDS-PAGE profiles of peroxisomal membranes may reflect the relatively low abundance of assembly factors in general. The membranes of *Candida boidinii*

peroxisomes contain a relatively abundant integral membrane protein of 47 kDa, as well as integral proteins of 32 and 31 kDa and a peripheral membrane-associated protein of 20 kDa (Goodman *et al.*, 1990; Goodman *et al.*, 1992). The peroxisomal membranes of *C. tropicalis* are more similar to those of *S. cerevisiae* with respect to protein profile showing three major integral polypeptides of 34, 29, and 24 kDa (Nuttley *et al.*, 1990).

An ATPase with possible proton-translocating properties has been detected both biochemically (Douma *et al.*, 1987) and immunocytochemically (Douma *et al.*, 1990c) in peroxisomes of the yeast *H. polymorpha*. ATPase activity has also been associated with the peroxisomes of rat liver (Wolvetang *et al.*, 1990). The role of this activity is not known, although it may function in the acidification of the peroxisomal matrix as outlined below.

Studies aimed at exploring the *in vivo* pH of the peroxisomal matrix by ^{31}P NMR (Nicolay *et al.*,1987) or by the accumulation of an immunocytochemically detectable weak base (Waterham *et al.*, 1990) agree that the peroxisomal membrane can support a pH gradient that is sensitive to treatment by proton ionophores. *In vivo* import and assembly of *C. boidinii* alcohol oxidase is inhibited by the proton ionophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP; Bellion and Goodman, 1987) suggesting that the proton gradient is essential for some aspect of protein translocation as is the case for *in vivo* precursor import into yeast mitochondria (Reid and Schatz, 1982).

In contrast to these results, membranes of isolated peroxisomes appear to be quite

permeable. Proteoliposomes incorporating peroxisome membrane fractions are permeable to small molecules such as sucrose and cannot maintain the pH gradient observed *in vivo* (Douma *et al.*, 1990b). Furthermore, proteins which can be shown to reside within the peroxisomal matrix immunocytochemically can leak extensively during organelle isolation procedures (McCammon *et al.*, 1990; Thompson and Krisans, 1990). In spheroplasts that are swollen osmotically, peroxisome proteins appear to remain confined to the peroxisomal matrix until the plasma membrane is disrupted (Douma *et al.*, 1990b).

Peroxisomes isolated from mammalian sources are freely permeable with respect several small molecules and cofactors (Van Veldhoven *et al.*, 1983), and this permeability appears to be associated with a 22 kDa peroxisomal membrane protein when it is incorporated into proteoliposomes (Van Veldhoven *et al.*, 1987). Another group has also studied the pore-forming capabilities of the mammalian peroxisomal membrane and also detected large cation selective pores (Labraca *et al.*, 1986).

Taken together, the results described above suggest that in intact cells, the peroxisomal membrane is an effective biological membrane that serves as a barrier to small molecules, but that the membrane is extremely sensitive to isolation conditions. The permeability characteristics of the isolated organelle do not reflect the *in vivo* characteristics of peroxisomes. Furthermore, it is difficult to assess the relevance of the pore-forming activity of some isolated proteins, when the *in vivo* functions of these proteins are completely unknown. The inability to isolate peroxisomes displaying the same permeability characteristics as the *in situ* organelle has important implications for

the development of robust *in vitro* systems for the study of peroxisome biogenesis and protein targeting.

1.5. METABOLIC FUNCTION OF PEROXISOMES

The function of peroxisomes can be stated most generally as the oxidative metabolism of most, if not all, types of nutrient substrates. The peroxisome appears to be a remarkably flexible organelle in that, at least in some yeasts, the enzyme complement of the matrix can be altered to enhance utilization of available substrates (Goodman *et al.*, 1990). In the interest of brevity this discussion will concentrate on examining peroxisome function in *S. cerevisiae*. The function of peroxisomes in humans (Mannaerts and Van Veldhoven, 1989, 1993; Van den Bosch *et al.*, 1992) and yeasts (Veenhuis and Harder, 1987), glyoxysomes in plants (Tolbert, 1981; Gietl, 1992; Kindl, 1993), and glycosomes in trypanosomatidae (Opperdoes, 1987) have been thoroughly reviewed elsewhere.

The primary biochemical function of peroxisomes in *S. cerevisiae* is the oxidation of fatty acids during growth on oleic acid. In mammalian cells peroxisomes are involved in the metabolism of very long-chain fatty acids (VLCFA) while another parallel pathway functions in mitochondria with specificity for shorter chain substrates. In yeasts however, the peroxisome appears to be the sole organelle involved in the utilization of fatty acids based on the detection of β-oxidation enzyme activities exclusively within these organelles (Kawamoto *et al.*, 1978; Kunau *et al.*, 1988).

The major metabolic pathways of *S. cerevisiae* peroxisomes are summarized in Figure 1. In *S. cerevisiae* fatty acyl-CoA oxidase is encoded by the *POX1* gene (Dmochowska *et al.*, 1990). In the more familiar mitochondrial β-oxidation pathway the flavin-adenine dinucleotide (FAD) cofactor of the fatty acyl-CoA dehydrogenase is regenerated by contributing electrons via intermediate electron transfer proteins to the mitochondrial electron transport chain, ultimately driving ATP formation (Stryer, 1988). In contrast, the FAD cofactor of peroxisomal fatty acyl-CoA oxidase is regenerated by reaction with molecular oxygen producing H_2O_2. H_2O_2 produced as the byproduct of β-oxidation cannot be allowed to accumulate to intracellular levels at which oxidative damage to DNA or other essential components occurs. There are two catalases in *S. cerevisiae*-- a peroxisomal catalase encoded by *CTA1* (Cohen *et al,*. 1988) and a cytosolic form encoded by *CTT1* (Hartig and Ruis, 1980). Deletion of both catalases does not result in a growth defect in strains grown on oleic acid probably because there is a marked increase in mitochondrial cytochrome *c* peroxidase activity (van der Klei *et al.*, 1990) and because growth of *S. cerevisiae* on oleic acid as the sole carbon source is too slow for peroxide-induced cytotoxicity to become apparent. In catalase-deficient strains growing on carbon sources in addition to fatty acids growth defects are more obvious probably because rapidly dividing cells are more sensitive to damage (Zhang *et al.*, 1993).

As in other peroxisomal β-oxidation pathways, a multifunctional enzyme catalyzes the next reactions. A significant difference between the *S. cerevisiae* β-oxidation pathway

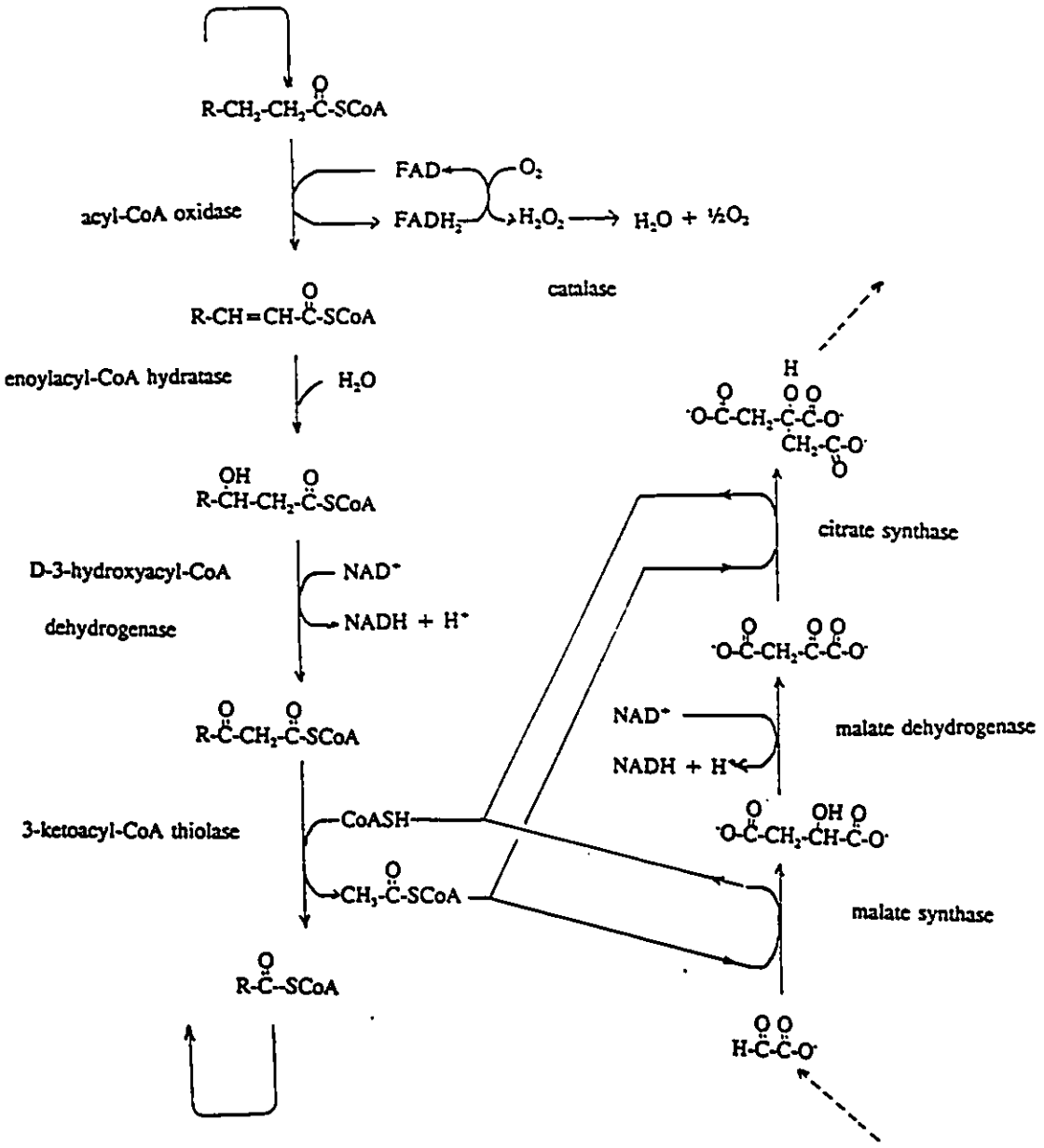

Figure 1. Schematic representation of some metabolic pathways in *S. cerevisiae* peroxisomes. The metabolic scheme is based on the peroxisomal proteins which are currently known to be exclusively peroxisomal or have peroxisomal isoenzymes in *S. cerevisiae*.

and other yeast peroxisomal β-oxidation schemes is the apparent absence of epimerase activity in the multifunctional protein encoded by *FOX2* (Hiltunen *et al.*, 1992). The substrate of the dehydrogenase is the D- rather than the L-isomer of the enoyl-CoA derivative (Hiltunen *et al.*, 1992).

The final step in each turn of the β-oxidation spiral is catalyzed by 3-ketoacyl-CoA thiolase encoded by the *POT1* gene in *S. cerevisiae* (Igual *et al.*, 1991). The gene was independently cloned by another group which used the designation *FOX3* (Einerhand *et al.*, 1991).

In addition to the enzymes of fatty acid oxidation, several enzymes of the glyoxylate pathway can be found associated with peroxisomes in *S. cerevisiae* (McCammon *et al.*, 1990). These include malate synthase which was detected by immunocytochemistry (McCammon *et al.*, 1990) and an isozyme of which has been recently cloned (*MLS2*; Fernandez *et al.*, 1993) and shown to be identical to *DAL7*, a gene required for allantoin metabolism in *S. cerevisiae* (Yoo and Cooper, 1989). Other glyoxylate pathway enzymes present in *S. cerevisiae* peroxisomes are a malate dehydrogenase isozyme is encoded by *MDH3* (Steffan and McAllister-Henn, 1992) and citrate synthase isozyme encoded by *CIT2* gene (Lewin *et al.*, 1990).

1.6. PEROXISOMAL TARGETING SIGNALS

Two types of peroxisomal targeting signals have been identified to date, but many peroxisomal matrix and membrane proteins whose sequences are known do not display

either of these motifs (Subramani, 1992a,1992b; Aitchison *et al.*, 1992b; Osumi and Fujiki, 1990). The assembly of membrane proteins into peroxisomal ghosts in Zellweger syndrome (Santos *et al.*, 1988) suggests that matrix components and membrane components are assembled by independent pathways. Recent analysis of peroxisomal assembly mutants in *S. cerevisiae* suggests that matrix components are imported by at least three pathways which are individually blocked by defects in different genes (Zhang *et al.*, 1994).

1.6.1. TYPE 1 PEROXISOMAL TARGETING SIGNAL (PTS1)

The first peroxisomal targeting signal was identified in the luciferase protein of the American firefly, *Photinus pyralis,* in the laboratory of Dr. Suresh Subramani (Gould *et al.*, 1989). During an effort to characterize the expression of luciferase in mammalian cells as a tool to study gene regulation, a punctate immunofluorescence staining pattern was observed in cells expressing the protein (de Wet *et al.*, 1987). The pattern of luciferase immunofluorescence overlapped precisely with catalase immunofluorescence, indicating a peroxisomal location (Keller *et al.*, 1987). It was subsequently shown that the C-terminal 12 amino acids of luciferase were required for targeting (Gould *et al.*, 1987). Similar short C-terminal segments of four other peroxisome matrix proteins were sufficient to direct a passenger protein into peroxisomes (Gould *et al.*, 1988), suggesting that the C-terminal location of targeting information might be a hallmark of peroxisomal matrix proteins .

Further analysis revealed that the C-terminal tripeptide Ser-Lys-Leu-COOH (SKL in single-letter code) was sufficient for peroxisomal targeting (Gould *et al.*,1989). Exploration of the acceptable redundancy within this tripeptide motif indicated that Ala and Cys could substitute for Ser, His and Arg for Lys, and that substitutions at, or extensions beyond, the C-terminal Leu abolished targeting (Gould *et al.*, 1989). Nevertheless, a study of all the permutations of these substitutions indicated that CHL was not a functional targeting signal (Swinkels *et al.*, 1992).

An anti-peptide antiserum raised against the C-terminus of luciferase recognised 15 to 20 proteins in rat liver peroxisomes but very few proteins in other organelles (Gould *et al.*, 1990a). Using this serum, conservation of SKL targeting across a wide range of diverse species was demonstrated (Keller *et al.*, 1991). Reaction of the antiserum with peroxisomes of mammals and yeast, glyoxysomes of plants, and glycosomes of trypanosomes is strong evidence for the homology of these organelles and for placing the origin of microbodies early in the eukaryotic ancestral line.

As with other organellar targeting signals, an array of naturally-occurring variations of the C-terminal tripeptide have been discovered. The C-terminal AKI of *C. tropicalis* multifunctional enzyme hydratase-dehydrogenase-epimerase (HDE) was shown to be necessary for targeting to peroxisomes in both *Candida albicans* (Aitchison and Rachubinski, 1990) and *S. cerevisiae* (Aitchison *et al.*, 1991). An antiserum generated against the HDE C-terminus recognized several *C. tropicalis* peroxisomal proteins while the anti-SKL serum detected few (Aitchison *et al.*, 1992a). In *S. cerevisiae* the

recognition pattern was reversed, with the anti-SKL serum detecting several peroxisomal proteins (see Table 1 for a list of *S. cerevisiae* proteins ending in SKL-COOH). Thus, even though the endogenous peroxisomal proteins of *S. cerevisiae* employ SKL-COOH signal which is immunologically distinct from AKI-COOH, AKI-COOH is nonetheless treated as an authentic targeting PTS1 and mediates import of the heterologously expressed HDE.

Table 1. Peroxisomal proteins of *S. cerevisiae* with SKL at C-terminus.

GENE	M_r(kDa)	NAME/FUNCTION	ANTI-SKL REACTIVITY[b]	REFERENCE
FOX2	98.4	multifunctional protein	yes	(1)
PAS6	68.2	peroxisome assembly factor	no	(2)
MLS2/DAL7[a]	62.8	malate synthase isozyme	yes	(3,4)
CIT2	54.0	citrate synthase isozyme	weak	(5)
MDH3	37.2	malate dehydrogenase isozyme	no	(6)

References (1)= Hiltunen *et al.*, 1992; (2)= Höhfeld *et al.*, 1992; (3)= Yoo and Cooper, 1989; (4)= Fernandez *et al.*, 1993; (5)= Lewin *et al.*, 1990; (6)= Steffan and McAllister-Henn, 1992. [a]Note that MLS2 and DAL7 are identical genes. [b]Detection by anti-SKL antiserum according to Aitchison *et al.*, 1992.

Other examples of SKL variants suggest that species differences in targeting are probably not unusual. The SKL variant SKI did not function as a peroxisomal targeting signal in mammalian cells (Gould *et al.*, 1989). Nonetheless, the C-terminal SKI of *H. polymorpha* catalase was required for peroxisomal targeting (Didion and Roggenkamp, 1992). In rat liver, the SKI at the C-terminus of epoxide hydrolase is thought to act as an impaired signal allowing the enzyme activity to be found both in the peroxisomal matrix and in the cytosol (Arand *et al.*, 1991). Import of phosphoglycerate kinase into glycosomes of *T. brucei* is directed by a C-terminal SSL (Sommer *et al.*, 1993), which

is not functional in mammalian cells (Gould *et al.*, 1989; Blattner *et al.*, 1992).

1.6.2. TYPE 2 PEROXISOMAL TARGETING SIGNAL (PTS2)

Studies of the biogenesis of peroxisomal enzymes suggest that the majority of peroxisomal matrix proteins are synthesized at their mature size on free polysomes (Goldman and Blobel, 1978; Roa and Blobel, 1983; Rachubinski *et al.*, 1984). The outstanding exception to this general rule is the peroxisomal 3-ketoacyl-CoA thiolase, which is synthesized as a larger precursor (Miura *et al.*, 1984; Fujiki *et al.*, 1985). In normal human cells the peroxisomal thiolase is found only in the processed form. However, cells of patients with Zellweger syndrome or rhizomelic chondrodysplasia punctata (see section 1.7 for descriptions of these conditions) accumulate only the precursor form of the protein (Balfe *et al.*, 1990; Schram *et al.*, 1986). The presence of the full-length protein in particulate form (peroxisomal ghosts) in cells without normal peroxisomes suggests that cleavage of the presequence is dependent on normal peroxisome assembly but is not required for translocation of the protein into the peroxisome matrix.

The presequences of many proteins destined to other organelles including the endoplasmic reticulum, mitochondria, and chloroplasts contain targeting information. The targeting activity of rat thiolase was examined in transfected mammalian cells using an immunofluorescense microscopy assay (Swinkels *et al.*, 1991). Mature thiolase remained cytosolic, while a fusion between the presequence and *Escherichia coli* chloramphenicol

acetyltransferase correctly redirected the cytosolic passenger protein to peroxisomes. The smallest segment of rat thiolase B (=thiolase 1) capable of peroxisomal targeting was the N-terminal 9 amino acids. Similar results were obtained independently by another group (Osumi *et al.*, 1991).

One interesting feature of peroxisomal thiolase biogenesis in rat is that there are two differentially regulated forms which are nearly identical over the length of the mature protein (Bodnar and Rachubinski, 1990; Hijikata *et al.*, 1987, 1990). Expression of the rPT-B (THI1) gene is induced by treatment of animals with hypolipodemic drugs, whereas the rPT-A (THI2) gene is constitutively expressed and unresponsive to drug. The major structural difference between the two proteins is that thiolase A has an additional 10 amino acid extension at its N-terminus compared to thiolase. Since both proteins are peroxisomal, the possibility exists that the N-terminal peroxisomal targeting signal need not reside at the extreme N-terminus. In contrast, PTS1 motifs do not appear to function at internal locations.

Table 2 shows a comparison of the N-terminal sequences of several peroxisomal thiolases and one plant glyoxysomal malate dehydrogenase isoenzyme that shares some features of the PTS2 of rat peroxisomal thiolase.

TABLE 2. Comparison of N-terminal sequences of peroxisomal proteins with putative PTS2 motif.

Species	N-terminal sequence	Ref
Rat A	MSESVGRTSAM-HRLQVVLG-HLAGRPESSSALQAAPC↓	1
Rat B	M-HRLQVVLG-HLAGRSESSSALQAAPC↓	2
Human	M-QRLQVVLG-HLRGPADSGWMPQAAPC↓	3
S. cerevisiae	MSQRLQSIKD-HLVLSAMGLGESKRKNSLLEK	4
C. tropicalis	M-DRLNQLSG-QLKPNAKQSILQKNPDDVVIV	5
Y. lipolytica	M-DRLNNLAT-QLEQNPAKGLDAITSKNPDDV	5
cucumber	MEKINR-QSILLHHLRPSSSAYTNESSLSASVC↓	6
cvmdh	MQPIPDVNQRIARISA-HLHPPKSQMEESALRRANC↓	7
	RLQ-V---HL	
consensus	N L Q	
	I	

The sequences are aligned to highlight residues which possibly correspond to a proposed consensus sequence given at bottom of table (bold in each sequence). The underlined residues highlight a conserved hydrophobic residue between the proposed consensus elements. Dashes are placed in sequences to enhance alignment. The downward pointing arrows correspond to known cleavage sites. The top 7 sequences are from peroxisomal 3-ketoacyl-CoA thiolases. The last sequence (cvmdh) is from watermelon glyoxysomal malate dehydrogenase. 1 = Hijikata *et al.*, 1987. 2 = Bodnar and Rachubinski, 1990. 3 = Bout *et al.*, 1988. 4 = Igual *et al.*, 1991. 5 = cited in de Hoop and Ab, 1992. 6 = Preisig-Müller and Kindl, 1993. 7 = Gietl, 1990.

1.7. HUMAN GENETIC DISORDERS OF PEROXISOME BIOGENESIS

Several clinical conditions in humans appear to arise from defects in peroxisome function and can be divided into 3 groups (Table 3).

Table 3. Classification of human peroxisomal disorders

Group 1: peroxisomes reduced or absent; multiple enzyme defects	Group 2 : peroxisomes normal; single enzyme defect	Group 3: peroxisomes present but structure abnormal; more than one enzyme defect
Zellweger syndrome	X-linked adrenoleukodystrophy	Rhizomelic chondrodysplasia punctata
Neonatal adrenoleukodystrophy	Acatalasemia	
Infantile Refsum disease	Hyperoxaluria type I	
Hyperpipecolic acidemia	Thiolase deficiency	
	Acyl-CoA oxidase deficiency	
	Bifunctional enzyme deficiency	

Adapted from Moser *et al.*, 1991

Group 1 disorders involve catastrophic defects in peroxisome assembly and are typified by Zellweger syndrome (Santos *et al.*, 1992; Schram *et al.*, 1986). The physiological manifestations of defective peroxisomal assembly are numerous and vary widely in severity (Mannaerts and Van Veldhoven, 1989; Moser *et al.*, 1991). However, the clinical hallmark of peroxisome assembly disorders is elevated serum levels of very long-chain fatty acids (VLCFAs) which are normally metabolized by mammalian peroxisomes. The manifestation at the subcellular level is the mislocalization of peroxisomal proteins and/or enzyme activities to the cytosol (Tager *et al.*, 1985; van Roermund *et al.*, 1991). Immunological reagents directed toward the peroxisomal membrane components allow the detection in Zellweger patient fibroblasts, of intact peroxisomal membranes which appear to lack the electron-dense components of the peroxisomal matrix (Santos *et al.*, 1988). This observation suggests that the underlying defect(s) of this group of disorders impairs the import of peroxisomal matrix proteins while assembly of membrane proteins is largely unperturbed. Biochemical analysis of the subcellular distributions of matrix proteins (Santos *et al.*, 1992) and complementation studies in which fibroblasts of patients are fused and the heterokaryons analyzed by immunofluorescence (Roscher *et al.*, 1989; Yajima *et al.*, 1992) have shown that severity and diagnosis of Zellweger syndrome and other peroxisome assembly disorders is highly influenced by the genetic background against which a given genetic defect is displayed.

A second group of disorders appears to arise from single enzyme deficiencies.

An interesting example of this group of disorders is primary hyperoxaluria type 1 which results from point mutations which redirect alanine:glyoxylate aminotransferase from peroxisomes to mitochondria (Danpure, 1993).

Group 3 disorders result from the mislocalization of a subset of peroxisomal proteins. Rhizomelic chondrodysplasia punctata is characterized by the mislocalization of peroxisomal 3-ketoacyl-CoA thiolase, acyl-CoA: dihydroxyacetone phosphate acyl transferase, and alkyldihydroxyacetone phosphate synthase (Hoefler *et al.*, 1988; Balfe *et al.*, 1990; Heikoop *et al.*, 1990). VLCAF-CoA synthetase activity is deficient in X-linked adrenoleukodystrophy, and it now appears that the enzyme fails to assemble into peroxisomes in at least some patients with the disorder (Aubourg *et al.*, 1993). The mislocalization of one or perhaps a limited subset of peroxisomal matrix components suggests that the import pathway specific to these proteins can be blocked without affecting the localization of the majority of peroxisomal proteins.

Cytosols of Zellweger fibroblasts from 6 complementation groups is capable of supporting peroxisomal localization of SKL-COOH peptides conjugated to human serum albumin (HSA) in a cytosol-dependent permeabilized cell system (Wendland and Subramani, 1993b), while normal cytosol fails to support import into the peroxisomal ghosts present in Zellweger fibroblasts. This result suggests that the failure to import matrix proteins in Zellweger syndrome arises from defects associated with the organellar membrane and that import factors that may be present in the

cytosol are not impaired in this disorder.

1.8. PEROXISOME ASSEMBLY FACTORS IN MAMMALIAN CELLS

Three genes have been cloned which are implicated in peroxisome biogenesis in humans. The first gene was originally found by complementation of Chinese hamster ovary mutant cell line, Z65, with a cell line harbouring a peroxisome assembly defect mimicking that of Zellweger syndrome cells (Tsukamoto *et al.*, 1991). The rat PAF-1 gene encodes a 35 kDa integral membrane protein with two predicted membrane-spanning domains. The protein is sensitive to external protease added to intact peroxisomes, indicating that the bulk of the protein is exposed to the cytosol. Intraspecies fusions between Z65 cells and cells from Zellweger patients were used to identify a human cell line in the same complementation group from which the human PAF-1 homologue was cloned and the genetic lesion determined at the level of DNA sequence (Shimozawa *et al.*, 1992).

The second mammalian peroxisome assembly gene was initially cloned from rat and encodes a 70 kDa integral membrane protein that is a member of the ATP-binding cassette (ABC) transporter family which includes P-glycoprotein associated with multidrug resistance and the chloride pump associated with cystic fibrosis (Kamijo *et al.*, 1990). Mutations in the human gene for PMP 70 have been found in a subset of Zellweger syndrome patients (Gärtner *et al.*, 1992).

X-linked adrenoleukodystrophy (ALD) is a disease characterized by

progressive demylelination of the central nervous system caused by accumulation of VLCFAs. VLCFA-CoA is metabolized normally in cells from patients with the disorder, indicating that, with the exception of fatty acid activation, β-oxidation in these patients is intact. A gene encoding a 35 kDa protein with significant similarity to PMP 70 is partially deleted in some ALD patients. It has been proposed that this protein is involved in the import or anchoring of VLCFA-CoA synthetase (Aubourg *et al.*, 1993).

1.9. PEROXISOME ASSEMBLY IN YEAST

The ability to generate mutant mammalian cell lines which mimic various human genetic disorders is an important contribution to the understanding of peroxisome biogenesis. However, the general pattern of organelle biogenesis may be well conserved among most eukaryotes. The identification of genes involved in peroxisome biogenesis in simpler unicellular eukaryotes such as yeast can provide valuable insight into the homologous processes in higher organisms.

In yeasts, metabolic pathways requiring peroxisomes are inducible, and negative screens (inability to grow on carbon sources requiring peroxisomes) capable of detecting peroxisomal defects are easily established. This strategy has been applied to several species of yeasts including *S. cerevisiae* (Erdmann *et al.*, 1989), *Pichia pastoris* (Gould *et al.*, 1992; Liu *et al.*, 1992) *H. polymorpha* (Cregg *et al.*, 1990; Waterham *et al.*, 1992; Titorenko *et al.*, 1993) and *Yarrowia lipolytica* (Nuttley *et*

al., 1993). Yeast strains derived from a mutagenized stock which are unable to grow on a carbon source requiring peroxisomal metabolism can be further characterized by one or more techniques, including cell fractionation and immunofluorescence microscopy. In the former case, peroxisome assembly mutants are identified by the cytosolic localization of enzymes normally associated with the organellar fraction. In the latter case, diffuse rather than punctate staining in immunofluorescence experiments using antibodies against peroxisomal proteins is indicative of impaired import. The genetic defect is cured by transformation of the mutant strain with a library of genomic DNA fragments from which the complementing gene can eventually be identified.

In addition to the negative screen outlined above, positive selection procedures have been used to find assembly mutants. One procedure exploited the sensitivity of normal cells to H_2O_2 generated by β-oxidation during growth on fatty acid when catalase activity is inhibited by 3-aminotriazole (van der Leij *et al.*, 1992). Mutants in which β-oxidation is impaired or absent are spared from peroxide-induced damage. Another screen based on the same idea (H_2O_2 toxicity), but using a catalase-deficient parental strain was recently reported (Zhang *et al.*, 1993a). A second and quite elegant positive screen used a luciferase-bleomycin resistance gene fusion that conferred resistance more readily in mutants that fail to sequester the resistance factor in the peroxisome matrix than in cells with normal peroxisomes (Elgersma *et al.*, 1993). In all, 17 complementation groups have been isolated in *S. cerevisiae*,

indicating that assembly of peroxisomes is a genetically complex event.

Peroxisome assembly (*pas*) mutants in *S. cerevisiae* (for reviews see Kunau *et al.*, 1993; Höhfeld *et al.*, 1992; Kunau and Hartig, 1992) can be divided into three classes. Type I *pas* mutants have no detectable peroxisome structures. Type II mutants have a few small peroxisomes, reminiscent of uninduced yeast and likely represent mutants in which factors required for induction of peroxisomes are impaired or absent. Type III mutants fail to assemble certain components but contain peroxisomes which appear to be otherwise normal. To date, the descriptions of five complementing genes in *S.cerevisiae* have been published in detail.

PAS1 encodes a 117 kDa protein with two conserved domains identifying it as a member of a growing superfamily of ATP-binding proteins (Erdmann *et al.*, 1991). Kunau *et al.* (1993) have suggested that this family be named the AAA family of proteins (ATPases associated with diverse cellular activities). Other members of this family include Sec18p of yeast and mammalian NSF, which are involved in membrane fusion events of the secretory pathway. A recent addition to this family is the *MSP1* gene of *S. cerevisiae*, whose product is located on the mitochondrial outer membrane and whose overexpression affects intramitochondrial protein sorting (Nakai *et al.*,1993). *PAS1* homologues have been identified in *Y. lipolytica* (*PAY4*; Nuttley *et al.*, 1993; Nuttley *et al.*, 1994) and *P. pastoris* (*PAS5*; Spong and Subramani, 1993).

The recently published sequence of the *PAS8* gene of *S. cerevisiae* (not to be

a member of the AAA family of ATPases with only one conserved nucleotide binding motif (Voorn-Brouwer *et al.*, 1993). Interestingly, the assembly mutant that contains the *pas8* mutation was discovered in a collection of "wild type" laboratory strains of *S. cerevisiae* (van der Leij *et al.*, 1992).

PAS3 encodes a 48 kDa integral peroxisomal membrane protein with no sequence similarity to other known proteins (Höhfeld *et al.*, 1991). The bulk of the protein is exposed to the cytosol as determined by protease digestion. Pas3p is not related to the 47 kDa PMP of *C. boidinii* and is not observed in the protein profile of purified peroxisome membranes of *S. cerevisiae* on stained gels (McCammon *et al.*, 1989).

PAS2 encodes a protein of 21 kDa, the sequence of which places the protein in the UBC (ubiquitin-conjugating) family of proteins (Wiebel and Kunau, 1992). A β-galactosidase-pas2p fusion protein comigrated with intact peroxisomes (density=1.22 $g \cdot cm^{-3}$) on sucrose density gradients and with Pas3p when expressed in an uncomplemented Type I *pas* mutant strain (density=1.14$g \cdot cm^{-3}$), suggesting that it is associated with peroxisomal membranes even in strains which do not assemble matrix components. A *PAS2* gene altered by site-directed mutagenesis to alter a conserved cysteine required for ubiquinating activity of other UBC proteins was unable to complement the Type I *pas2* phenotype suggesting that ubiquination provides some type of signal required for assembly (Wiebel and Kunau, 1992).

As opposed to the general assembly defects described in the above mutants,

the *pas8* mutant of *P. pastoris* is capable of importing thiolase, whereas other enzymes of the peroxisomal matrix are cytosolic (McCollum *et al.*, 1993) including proteins which react with anti-SKL antiserum. The phenotype of *pas*8 suggests that components of the import pathway mutually required by proteins with either PTS1 or PTS2 targeting motifs remain intact (indicated by thiolase import) but that a component required exclusively by PTS1-targeted proteins is defective. Consistent with this view, *PAS8* encodes a 65 kDa (possibly integral peroxisomal membrane) protein which is capable of binding a synthetic PTS1 peptide *in vitro* (McCollum *et al.*, 1993) and may be the putative PTS1 receptor molecule. The protein encoded by *PAS8* contains 7 imperfect repeats of a 34 amino acid sequence identifying it as a member of the TPR (tetratricopeptide repeat) family of proteins. The *pay*32 mutant of *Y. lipolytica* exhibits a phenotype identical to that of *P. pastoris pas*8. Sequencing results indicate that the gene complementing *pay*32 also encodes TPR domains and that it is likely a *PAS8* homologue (Rachel K. Szilard, personal communication). In *S. cerevisiae* the homologous defect is represented by the *pas*10 mutant, and the *PAS10* gene is also a TPR protein (Van Der Leij *et al.*, 1993).

1.10. FOCUS OF THIS THESIS

Analysis of the subcellular location of matrix proteins in both human genetic disorders and in yeast assembly mutants suggests that translocation of thiolase and the majority of other matrix components are genetically distinguishable even though most

assembly defects result in mislocalization of both. This suggests that translocation of PTS1- and PTS2-targeted proteins is initiated by separate events which probably funnel into a common complex translocation machine which is composed of several essential components.

The overall purpose of these experiments was to look at thiolase targeting in *S. cerevisiae*. The first goal was to establish whether the yeast thiolase is targeted to peroxisomes by a PTS2 type signal. The second goal was to examine the structural requirements of PTS2 in yeast. The third goal was to characterize what appears to be a novel pathway for protein targeting and translocation involving the formation of thiolase dimers in the cytosol prior to translocation of the oligomerized protein.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

agar	Difco
agarose	BRL
albumin (bovine serum)	Sigma
ampicillin	Sigma
antipain	Sigma
β-mercaptoethanol	BDH
BioRad protein assay	BioRad
cesium chloride	BRL
chymostatin	Sigma
Coomassie Brilliant Blue (R-250)	Gibco/BRL
cytochrome *c* (horse heart)	Sigma
dithiothreitol	Sigma
EDTA	Sigma
Ficoll	Sigma
GTG agarose	FMC BioProducts
hydrogen peroxide (30%)	Sigma
isopropyl β-D-thiogalactoside	Gibco/BRL
leupeptin	Sigma
L-leucine	Sigma
L-methionine	Sigma
L-histidine	Sigma
L-lysine	Sigma
MES	Sigma
nitrocellulose (pore size-0.45μm)	Schleicher and Schuell
Nycodenz	Nycomed
oleic acid	Fisher Scientific
ovalbumin	Miles
PANSORBIN	Calbiochem
pepstatin A	Sigma
peptone	Difco
salmon sperm DNA	Sigma
Sephadex G-50 (medium)	Pharmacia
sodium dithionite	Sigma
titanium oxysulfate hydrate	Aldrich
Tris	BRL; Boehringer Mannheim

Triton X-100	BioRad
Tween 20	Sigma
Tween 40	Sigma
X-gal	GIBCO/BRL
X-ray film (X-AR)	Eastman Kodak
yeast extract	Difco
YNB (w/o amino acids)	Difco

2.1.2. ENZYMES

Trypsin	Sigma
Thermolysin	Sigma
Ribonulease A	Pharmacia
Zymolyase 100T	ICN
Zymolyase 20T	ICN
3-hydroxyacyl-CoA dehydrogenase	Boehringer Mannheim
lactate dehydrogenase	Boehringer Mannheim

2.1.3. DNA MODIFYING ENZYMES

Calf intestinal phosphatase	NEB
DNA ligase (T4)	Gibco/BRL; NEB; Promega
Klenow fragment of DNA polymerase I	NEB; Pharmacia; Gibco/BRL
polynucleotide kinase (T4)	Pharmacia
restriction endonucleases	Gibco/BRL; NEB; Pharmacia; Boehringer

2.1.4. MULTI-COMPONENT SYSTEMS

ECL	Amersham
rabbit reticulocyte lysate	Promega
random primers labelling system	Gibco/BRL
Sequenase DNA sequencing system	USB
In vitro transcription kit	Promega
Qaiex beads	Qaiex

2.1.5. MOLECULAR WEIGHT STANDARDS

1 kbp DNA ladder	Gibco/BRL
Prestained molecular weight markers	Bio-Rad
^{14}C-Molecular weight standards (14 to 70 kDa)	Sigma

2.1.6. IMMUNODETECTION REAGENTS

HRP-conjugated anti-rabbit IgG	Amersham
AP-conjugated anti-mouse IgG	Promega
AP-conjugated anti-rabbit IgG	Promega

2.1.7. RADIOCHEMICALS

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

2.2. RECOMBINANT DNA TECHNIQUES

2.2.1. BACTERIAL STRAINS AND CULTURE CONDITIONS

All plasmids were amplified and maintained in *E. coli* strain DH5α (*endA1, hsdR17*($r_K^-m_K^+$), *supE44, thi-1, recA1, gyrA,* (Nal^r) *relA1, Δ(lacZYA-argF)*$_{U169}$, (m80*lacZΔM15*); Hanahan, 1983). Competent cells were obtained from GIBCO/BRL and transformed according to the suppliers instructions. The transformed cells were grown in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and adjusted to pH 7.0 for addition of agar for plates) supplemented with 75 to 150 µg·mL^{-1} ampicillin.

2.2.2. PREPARATION OF PLASMID DNA

Plasmid DNA from small amounts of cells (1.5 mL) was prepared by alkaline lysis (Maniatus, 1982). Plasmids from 100 to 500 mL cultures were isolated by alkaline lysis and purified on cesium chloride gradients (Maniatis, 1982) or on QIAEX™ columns

according to the manufacturer's protocol.

2.2.3. RESTRICTION ENDONUCLEASE DIGESTIONS

Restriction digests were performed according to the manufacturers' instructions in supplied buffers or according to Hanish and McLelland (1988) in KGB buffer (5 X KGB = 100 mM potassium glutamate, 25 mM Tris-actetate pH 7.6, 10 mM magnesium acetate, 0.5 mM 6-mercaptoethanol, 50 $\mu g \cdot mL^{-1}$ BSA). Digestion products were analyzed by electrophoresis in 1 to 4% agarose gels run in either TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) or TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) containing 20 $\mu g \cdot mL^{-1}$ ethidium bromide.

2.2.4. PURIFICATION OF DNA FRAGMENTS

Fragments resulting from restriction endonuclease digestion were purified by either of the following methods.

Electroelution

Restriction digestion products were separated on 1% Genetic Technology Grade™ agarose (FMC BioProducts) gels run in 1 x TBE. Bands for purification were excised and placed in the well of an electroelution apparatus (IBI). The chambers were filled with 0.5 X TBE and the "elbows" were filled with 80 µL of 7.5 M ammonium acetate with 0.1 % bromophenol blue. After the DNA was completely eluted from the gel slice, 2 X 175 µL aliquots were recovered from the elbow and precipitated with 1 mL absolute

ethanol and 25 µg linear polyacrylamide as a carrier (Gaillard and Strauss, 1990).

QIAEX™ beads

Restriction digestion products were separated on 1% electrophoresis grade agarose gels run in 1 x TAE. Gel slices were solubilized, and the DNA, bound to a solid support material, was recovered exactly according to the manufacturer's instructions.

2.2.5. GENERATION OF BLUNT-ENDED DNA

As required, 5'-overhangs generated by restriction endonuclease digestion were filled with the Klenow fragment of DNA polymerase in the presence of 100 µM dNTPs at 37°C for 20 min (Ausubel *et al.*, 1987). 3'-overhangs were polished by treatment with T4 DNA polymerase I in the presence of 100 µM dNTPs at 11°C for 20 min (Ausubel *et al.*, 1987). Both enzymes were inactivated by heating at 75°C for 15 min.

2.2.6. LIGATION OF DNA FRAGMENTS

The concentrations of purified DNA fragments was estimated on ethidium bromide stained agarose gels by visual comparison of band intensities with the intensity of molecular size standards (1kbp ladder, BRL) of known concentration. Ligations of fragments with distinct compatible ends were performed in 0.5 X KGB, 1 mM ATP, 1 U T4 DNA ligase with 10 to 50 ng of vector and a 2- to 3-fold molar excess of insert in 10 µL reaction at room temperature. When a restriction fragment with the same 3' and 5' ends was to be inserted into a linearized vector, the vector was treated with calf

intestinal phosphatase (CIP) to reduce unproductive reclosing of the vector upon itself. For blunt-blunt ligations, vector fragments were treated with CIP and 100 to 200 ng of vector was ligated together with a 5-fold molar excess of insert, 0.1 mM ATP and 5 U of ligase at 16°C overnight.

2.2.7. SEQUENCING OF DNA

Sequencing reactions were performed on double stranded templates (Zhang *et al.*, 1988) by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase II™ system (United States Biochemicals) with [α-^{32}P]-dATP to label. Reactions were run on 5% Long Ranger™ gels (J.T. Baker). A single loading was satisfactory for reading sequences required in these experiments. Gels were dried onto Whatman 3MM paper and exposed to X-ray film overnight at room temperature.

2.2.7. LABELLING OF DNA PROBES

DNA fragments were radiolabelled with [α-^{32}P]-dATP using a random primers labelling kit (BRL) and the Klenow fragment of DNA polymerase I. Unincorporated labelled nucleotide was removed by centrifugation over a column of Sephadex G-50 in TE pH 8.0 with a bed volume of 1 mL (Maniatis *et al.*, 1982). Incorporation of label was assessed by liquid scintillation counting.

2.2.8. SOUTHERN BLOT ANALYSIS

Southern blot analysis (Southern, 1975) was used to detect diagnostic genomic DNA fragments in yeast strains with disrupted thiolase genes. Yeast strains were grown in YEPD (Table 3). Washed cells were converted to spheroplasts with zymolyase, and briefly lysed with glass beads in a buffer consisting of 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 1% SDS and 2% Triton X-100. Cell debris was removed by centrifugation, and the supernatant was extracted once with phenol/chloroform/isoamyl alcohol (50:49:1) and once with chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated with 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate pH 5. The pellet was dried and dissolved in TE 8.0 with 20 $\mu g \cdot mL^{-1}$ RNase A and incubated for 3 h at 37°C. The DNA solution was re-extracted and precipitated. The final pellet was redissolved in TE pH 8.0, and DNA content was determined by UV absorbance at 260 and 280 nm.

20 µg of yeast genomic DNA was subjected to restriction endonuclease digestion under conditions that favoured complete digestion. 5 µg of digested DNA was subjected to electrophoresis on 1% agarose. The gel was stained with ethidium bromide and photographed. The gel was exposed for 5 min on a UV transilluminator to nick DNA strands. The DNA was denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for 30 min followed by neutralization in a few changes of 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5. The DNA was transferred onto nitrocellulose by capillary action in 5 X SSC (1 X SSC = 15 mM trisodium acetate pH 7.0, 150 mM NaCl). DNA was fixed onto the

nitrocellulose by UV cross-linking (0.12 J·cm^{-2}) using a Stratalinker™ (Stratagene). Blots were probed as described in section 2.2.10.

2.2.9. COLONY HYBRIDIZATION

Occasionally, bacterial colonies were screened for the presence of recombinant plasmids essentially as described by Hanahan and Meselson (1980). Bacterial colonies were lifted onto nitrocellulose filters and placed, colony side up, on 1 mL puddles of 0.2 M NaOH (repeated once), 1 M Tris-HCl pH 7.5 (repeated once) and 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5 (repeated once). The filters were briefly washed in 1 X SSC to remove debris, and DNA was fixed to the nitrocellulose by UV crosslinking. Hybridization is described in the following section.

2.2.10. HYBRIDIZATION OF LABELLED PROBES

The method described was used for either Southern blot analysis or colony hybridization. Non-specific binding sites on nitrocellulose were blocked by incubation with hybridization solution (1.25 X SSC, 0.16 X Denhardt's solution [1 X Denhardt's solution = 0.1% Ficoll, 0.1% polyvinylpyrolidone, 0.1% BSA], 0.001% SDS, 20 mM sodium phosphate pH 7.0, 4 μg·mL^{-1} denatured sheared salmon sperm DNA) in a sealed bag for not less than 3 h at 65°C. Just prior to use, labelled probes were denatured by boiling for 10 min followed by rapid cooling on ice. The denatured probe was diluted to 1 x 10^{6} dpm·mL^{-1} in hybridization solution containing 30% (v/v) deionized formamide.

Hybridization was carried out at 50°C for not less than 3 h. Blots were washed for 4 X 10 min in 1 X SSC at 50°C. Hybridization was detected by exposure of the blot to X-ray film (Kodak) or to a phosphor storage screen (Molecular Dynamics).

2.3. PLASMIDS

2.3.1. pRS PLASMIDS

Low-copy number vectors (pRS313 and pRS315) include *CEN6* and *ARS4* sequences required for inheritance and replication in *S. cerevisiae* and selectable markers *HIS3* and *LEU2* respectively (Sikorski and Hieter, 1989). Specifically, *ARS*s (autonomously replicating sequences) contain origins of replication which function in yeast and allow the plasmid to be replicated during the S phase of mitosis. *CEN* (centromeric) sequences appear to be involved in inheritance of newly replicated plasmids during anaphase. High-copy number vectors (pRS423 and pRS425; Christianson *et al.*, 1992) contain sequences derived from the 2 μm circle. The 2 μm circle element confers a high-copy number phenotype with 20 or more copies of the plasmid per cell.

2.3.2. *S. CEREVISIAE* ACYL-CoA OXIDASE PROMOTER

It was desirable that the genes used in these studies would be expressed in a fashion is concomitant with the formation of the target organelle. All coding regions were constructed downstream from a promoter encompassing nucleotides -2 to -454 of the *POX1* gene which encodes *S. cerevisiae* peroxisomal acyl-CoA oxidase (Dmochowska

et al., 1990). This region includes two *cis*-acting elements which are required for full regulation of transcription of downstream sequences in response to growth on various carbon sources (Einerhand *et al.*, 1991; Wang *et al.*, 1992; Simon *et al.*, 1992). The distal element is a URS (upstream repressor sequence) at -423 to -430 with the sequence 5'-AGGGTAAT-3' and is primarily essential for repression of transcription during growth on glucose. The proximal element is a UAS (upstream activating sequence) with the sequence 5'-CGGCGATTA-3' corresponding to the consensus sequence 5'-CGGNNNTNA-3', which is required for full induction of transcription during growth on oleic acid.

2.3.3. pSG524 AND pSG522

These plasmids (Fig. 2) were a gift from Dr. Suresh Subramani and have not been described in detail elsewhere. The thiolase gene was amplified by the polymerase chain reaction (PCR) using the following synthetic oligonucleotides:

(A) 5'-GGGGATCCGCTAGCCATGTCTCAAAGACTACAAAGT-3'

(B) 5'-CCTCTAGACTCGAGAAATAATGAAAATGGAA-3'

The oligonucleotide A was designed to introduce *Bam*HI and *Nhe*I sites immediately upstream of the initiation codon and to convert some of the wild type T at position +4 (underlined in nucleotide A) to G, creating a novel *Nco*I site at the initiation codon. The oligonucleotide B was designed to introduce *Xho*I and *Xba*I sites at a location in the 3' untranslated region of the thiolase gene 119 bp downstream of the termination codon.

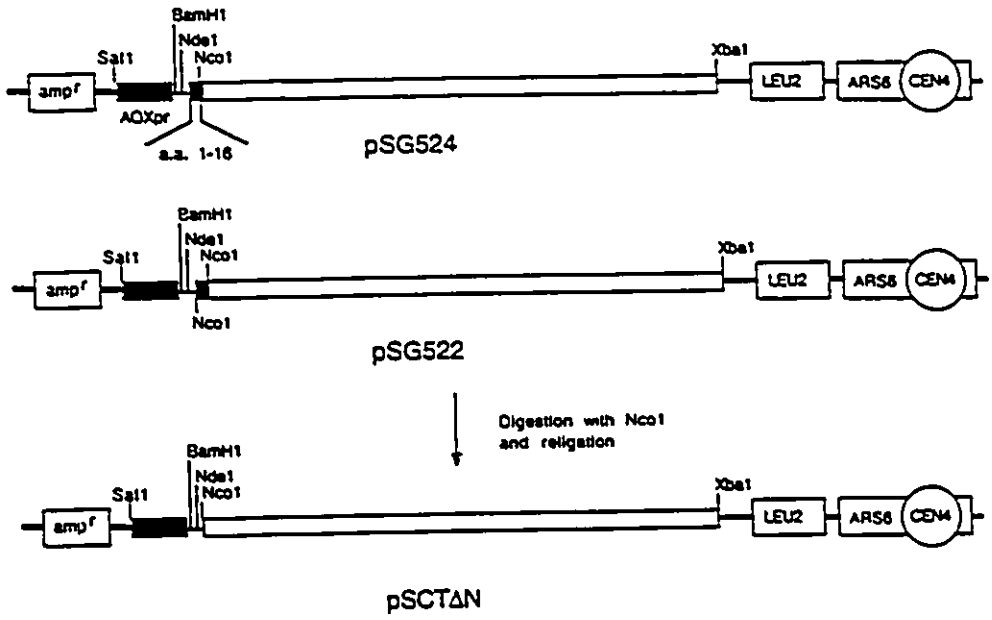

Figure 2. Structure of plasmids pSG524 and pSG522 and construction of pSCTΔN. The details of the construction of pSG522, pSG524, and pSCTΔN are provided in the text.

PCR products were subcloned as *Bam*HI-*Xba*I fragments into a vector consisting of a pRS315 backbone (*ARS4*, *CEN6*, *LEU2*) and the *AOX* promoter described above. pSG524 contains the unmodified thiolase coding region while pSG522 contains the thiolase coding region with the novel *Nco*I site at the initiation codon and which converts the second codon from one encoding Ser to one encoding Ala.

The structure of these two plasmids was well suited to the construction of passenger proteins to be expressed either as an unfused control or as a fusion with the N-terminal 16 amino acids of *S. cerevisiae* thiolase (SCT) containing the putative PTS2 targeting motif.

2.3.4. CONSTRUCTION OF pSCTΔN

The plasmid pSG522 was digested with *Nco*I and recircularized by ligation. This action creates a plasmid (Fig. 2) which encodes a thiolase which initiates translation at Met16 and was designated as pSCTΔN to signify that the thiolase encoded by this plasmid is N-terminally truncated.

2.3.5. DIHYDROFOLATE REDUCTASE-SCT N-TERMINUS FUSION

Plasmid 35-2 is based on pSP73 and contains the coding region of a modified mouse dihydrofolate reductase (DHFR) cDNA with a *Hinc*II site permitting the formation of a blunt-ended DNA fragment at nucleotide +6 (R.A. Rachubinski, personal communication). Plasmid 35-2 was digested with *Nco*I, end-repaired with Klenow

polymerase and religated to destroy this site in the 3'-untranslated region of the DHFR cDNA. Synthetic DNA linkers (NEB) with the sequence 5'-CAGCCATGGCTG-3' containing an *Nco*I site were ligated into the *Hinc*II site creating the plasmid pDHFRN. pSG524 and pSG522 were digested with *Hind*III and end-repaired with Klenow polymerase, then digested to completion with *Nco*I and the vector backbones purified. pDHFRN was digested with *Nco*I and *Sma*I, and the purified DHFR coding region (187 amino acid residues) was ligated into the prepared vectors. The recombinant plasmid derived from pSG524 was designated pDHFRSCTN-AOX, and the plasmid derived from pSG522 was designated pDHFRN-AOX (Fig. 3).

2.3.6. CHLORAMPHENICOL ACETYLTRANSFERASE-SCT N-TERMINUS FUSION

The plasmid YEpCAT-PGK is a YEp13-based plasmid with the *E. coli* chloramphenicol acetyltransferase (CAT) gene under the regulation of the *S. cerevisiae* phosphoglycerate kinase promoter (J.D. Aitchison, personal communication). The CAT gene was cloned into a *Bgl*II site in the *PGK* promoter construct as a *Bam*HI fragment including 33 bp of 5'-untranslated and 86 bp of the 3'-untranslated region of CAT. pSG524 and pSG522 were digested to completion with *Nco*I, end-repaired with Klenow polymerase, further digested with *Hind*III and the vector fragment purified. YEpCAT-PGK was digested with *Pvu*II and *Hind*III, and the fragment containing the coding sequence from the codon for Leu39 of CAT and including 450 bp of 3'-untranslated region of the *PGK* gene was purified. This insert was ligated into pSG524

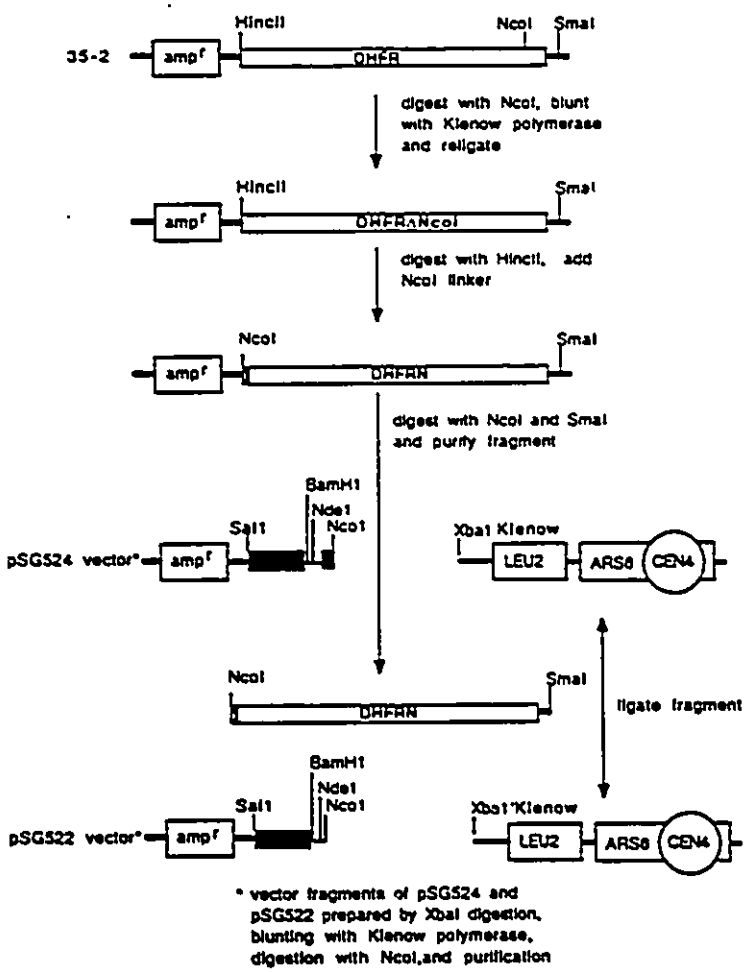

Figure 3. Construction of plasmids for the expression of DHFR and DHFR fused to the N-terminus of thiolase. Details of DNA manipulations are given in the text. The plasmid resulting from the insertion of the DHFRN fragment into the pSG522-derived vector was designated pDHFR.AOX and directs the oleic acid-inducible expression of the slightly modified DHFR domain. The plasmid derived from pSG524 was designated pDHFRSCTN.AOX and directs the expression of the DHFR domain fused to the N-terminal 16 amino acids of *S. cerevisiae* thiolase.

and was designated pCATSCTN-AOX. The construct fusing the truncated CAT gene to the start codon of SCT in pSG522 was designated pCATΔN-AOX (Fig. 4).

2.3.7. CONSTRUCTION OF PTS1-PROTEIN A FUSIONS

Construction of the three plasmids described in this section was done in collaboration with Dr. Fabiola Janiak working in the laboratory of Dr. David Andrews. Synthetic oligonucleotides with GATC 5'- overhangs (see below) were dissolved in water to a concentration of 0.25 mM. An aliquot of each oligonucleotide (250 nmol) was phosphorylated with polynucleotide kinase and ATP. Equal amounts of complimentary oligonucleotide pairs were heated to 75°C and slow cooled to room temperature to promote annealing. The plasmid pSPUTKgGPrA (Janiak *et al.*, 1994) was digested with *Bam*HI and dephosphorylated with calf intestinal phosphatase. Annealed duplexed oligonucleotides were ligated into the prepared plasmid. Recombinant plasmids were detected by restriction digest and then analyzed by dideoxy sequencing. The resulting plasmids (Fig. 5) were designated pgGPrASTOP (generated with oligonucleotides 5'-GATCTTATAGGCGGCGGCG-3' and 3-AATATCCGCCGCCGCCTAG-5'), pgGPrASKL (generated with oligonucleotides 5'-GATCTTATCCAAATTATAG-3' and 3'-AATAGGTTTAATATCCTAG-5'), and pgGPrAAKI (generated with oligonucleotides 5'-CATCTTAGCAAAAATCTAG-3' and 3'-AATGCTTTTTAGATCCTAG-5').

In order to express these genes in yeast, the plasmid pSG522 was digested to completion with *Xba*I and end-repaired with the Klenow fragment of DNA polymerase

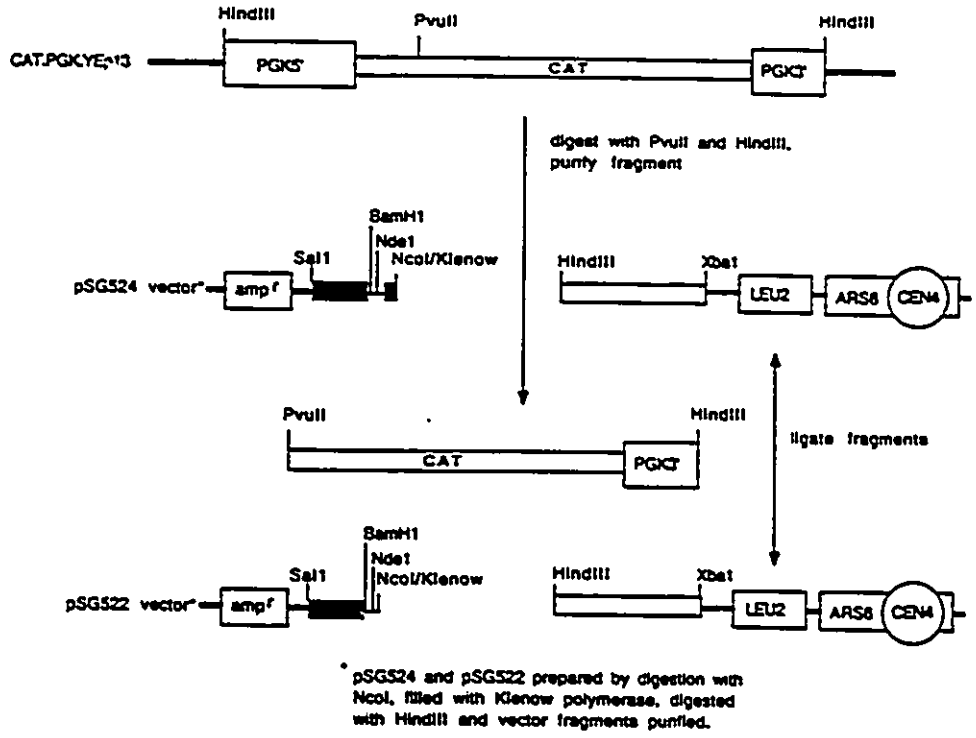

Figure 4. Construction of plasmids for the expression of CAT, truncated CAT, and CAT fused to thiolase N-terminus. Details of DNA manipulations are given in text. The plasmid pCATΔN·AOX directs the oleic acid-inducible expression of a truncated CAT with amino acids 1 to 38 deleted. The plasmid pCATSCTN·AOX directs the expression of the same CAT domain fused with the 16 N-terminal amino acids of *S. cerevisiae* thiolase.

I. The entire coding region of *S. cerevisiae* thiolase was eliminated by digestion with *Nco*I, and the large vector fragment was gel purified. The three plasmids described above were digested to completion with *Eco*RV and partially with *Nco*I and the fragments corresponding to the larger of the two *Nco*I-*Xba*I coding regions generated by the partial digest were purified and ligated to the prepared vector. Recombinants were checked for insertion of the entire gGPrA coding region by restriction digests. These plasmids (Fig. 5) were designated pgGPrASTOP·AOX, pgGPrASKL·AOX, and pgGPrAAKI·AOX to indicate that the fused and unfused passenger proteins would be expressed under the regulation of the *AOX* promoter.

2.3.8. PROTEIN A-SCT N-TERMINUS FUSION

A plasmid encoding gGPrA fused to the putative N-terminal PTS2 of SCT was constructed essentially as described in the section describing the subcloning of other gGPrA constructs into the yeast expression vector, except that the *Nco*I-*Eco*RV fragment of pgGPrASTOP was inserted into a vector derived from the plasmid pSG524. The resulting plasmid was designated pgGPrASCTN (Fig. 5).

Versions of the four plasmids encoding the gGPrA passenger protein domain but lacking the DNA encoding the 27 amino acids of glycoglobin were constructed by digesting each plasmid with *Nco*I and recircularizing the plasmid by ligation. These were designated as pPrASTOP·AOX, pPrASKL·AOX, and pPrAAKI·AOX.

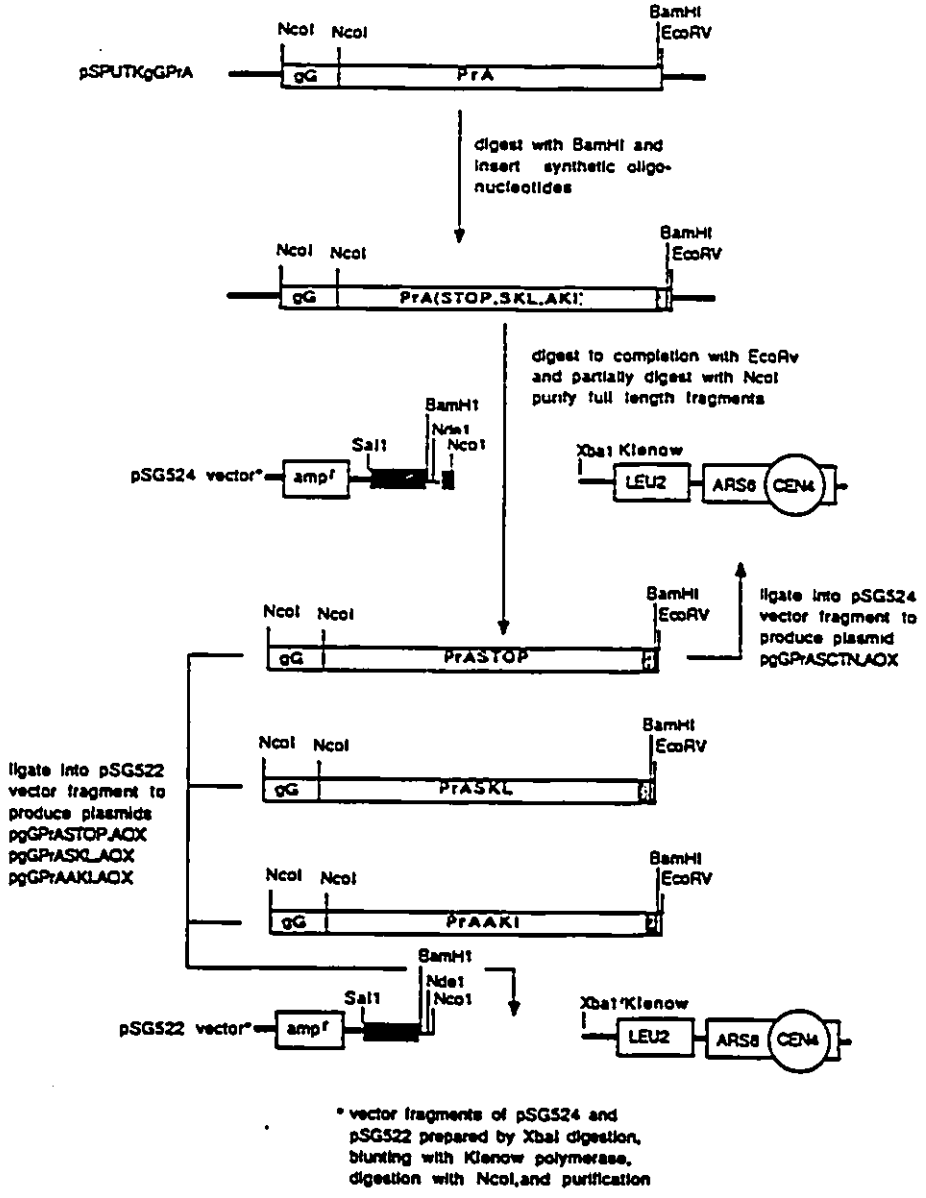

Figure 5. Construction of plasmids for the expression gGPrA. Details of DNA manipulations are given in the text. The plasmid pgGPrA-AOX directs the oleic acid-inducible expression of the glycoglobin/Protein A domain. Plasmids pgGPrASKL-AOX, pgGPrAAKI-AOX, and pgGPrASCTN-AOX are for the expression of the same domain fused to the PTS1 motif of firefly luciferase, *C. tropicalis* multifunctional protein, and the N-terminal 16 amino acids of *S. cerevisiae* thiolase respectively.

2.3.9. CONSTRUCTION OF PTS2 MUTANTS

The following synthetic oligonucleotide was obtained from Dalton Chemicals:

5'-CTTGGATCCGCTAGCCATGTCTCAAagactacaaAGTATCAATcattgGTGGAGAGCGCCATGGCG-3'

The lower case letters represent positions where, in addition to the wild-type base indicated, the synthesis reaction was "doped" with small amounts of each of the three other nucleotides so that the predicted average composition at each position would be 90% wild-type base and 3.3% of each of the other three bases. The underlined letters indicate a palindromic sequence of 10 nucleotides. The oligonucleotide was phosphorylated with polynucleotide kinase and ATP, heated to 100°C, slow cooled to room temperature and then further cooled to 4°C. The self-annealed oligonucleotide was extended overnight at 16°C with 5 U of Klenow fragment in the presence of 2.5 mM dNTPs and 10 μCi of [α-^{32}P]-dATP added as a tracer. Extension was continued for a further 2 h at room room temperature with fresh dNTPs and fresh polymerase. The reaction was extracted with phenol:chloroform:isoamyl alcohol and once with chloroform:isoamyl alcohol and precipitated with ethanol in the presence of 300 mM sodium acetate pH 5.0 and 10 μg linear polyacrylamide. The redissolved duplex was digested with an excess of *Bam*HI and applied to a 5% polyacrylamide slab gel. The gel was stained with ethidium bromide, photographed, and then exposed to X-ray film. The region around the 154 bp marker was excised and electroeluted. The resulting product was digested with *Nco*I.

In order to obtain the lowest possible level of background religation of

incompletely digested vector, a 2 kbp *Bam*HI-*Nco*I fragment of DNA was cloned into pSCTΔN between the *Bam*HI and *Nco*I sites of that vector (pSCT2kbi). This plasmid was digested with *Bam*HI and *Nco*I and gel purified.

The plasmid was ligated together with the *Nco*I-*Bam*HI digested oligonucleotide duplex. This action replaces the normal coding sequence of thiolase with the sequence generated by the synthetic oligonucleotide from immediately upstream of the initiation codon to base pair +47 of the thiolase coding region. All plasmids recovered from this ligation contained inserts derived from the synthetic oligonucleotides, and 192 clones were sequenced by the dideoxy method using the primer 5'-CAATAACTACATCTT-3' (Central Facility of the Institute of Molecular Biology, McMaster University) complementary to bases +100 to +115 of the thiolase coding region.

2.3.10. CONSTRUCTION OF EPITOPE-TAGGED SCT

A pair of complementary synthetic oligonucleotides (5'-CATGTACCCATACGACGTCCCAGACTACGCTGC-3' and 3'-ATGGGTATGCTGCAGGGTCTGATGCGACGGTAC-5' generating 5'-overhangs with the sequence CATG) were phosphorylated and annealed as described above and ligated to pSCTΔN that had been digested with *Nco*I and dephosphorylated using calf intestinal phosphatase. Recombinant plasmids were detected by restriction digestion and further characterized by dideoxy sequencing and the ability to program the expression in yeast of an epitope-tagged protein of the appropriate molecular size as determined by

immunoblotting of cell extracts.

2.4. YEAST METHODS

2.4.1. CULTURE CONDITIONS

The compositions of media used to culture various yeast strains are given in Table 3. Yeast strains not containing plasmids were maintained on YEPD agar. Yeasts transformed with plasmids were selected and maintained on YNBD agar appropriately supplemented (Rose *et al.*, 1988). YNO agar was used to assess growth on oleic acid as a carbon source (Erdmann *et al.*, 1989). YNO was also used as a liquid medium for cell-labelling experiments. SCIM (*Saccharomyces cerevisiae* Induction Medium) was used in experiments in which induction of peroxisomes and significant growth of the culture were required (Lewin *et al.*, 1990).

2.4.2. CONSTRUCTION OF THIOLASE-DEFICIENT STUD STRAIN

In order to express SCT PTS2 mutants in a background deficient in endogenous thiolase, a *pot1*::*URA3* construct was made (Fig. 6) and used to obtain the STUD (*Saccharomyces* Thiolase *URA3* Disruption) strain by homologous recombination. A 1040bp *Hinf*I fragment of the *URA3* gene, including 154 bp of sequence upstream and 81 bp downstream of the open reading frame was excised from YCp50, made blunt with Klenow fragment and subcloned into the *Sma*I site of pGEM7Zf(+). Plasmids containing the *URA3* gene in both orientations were recovered and were designated as

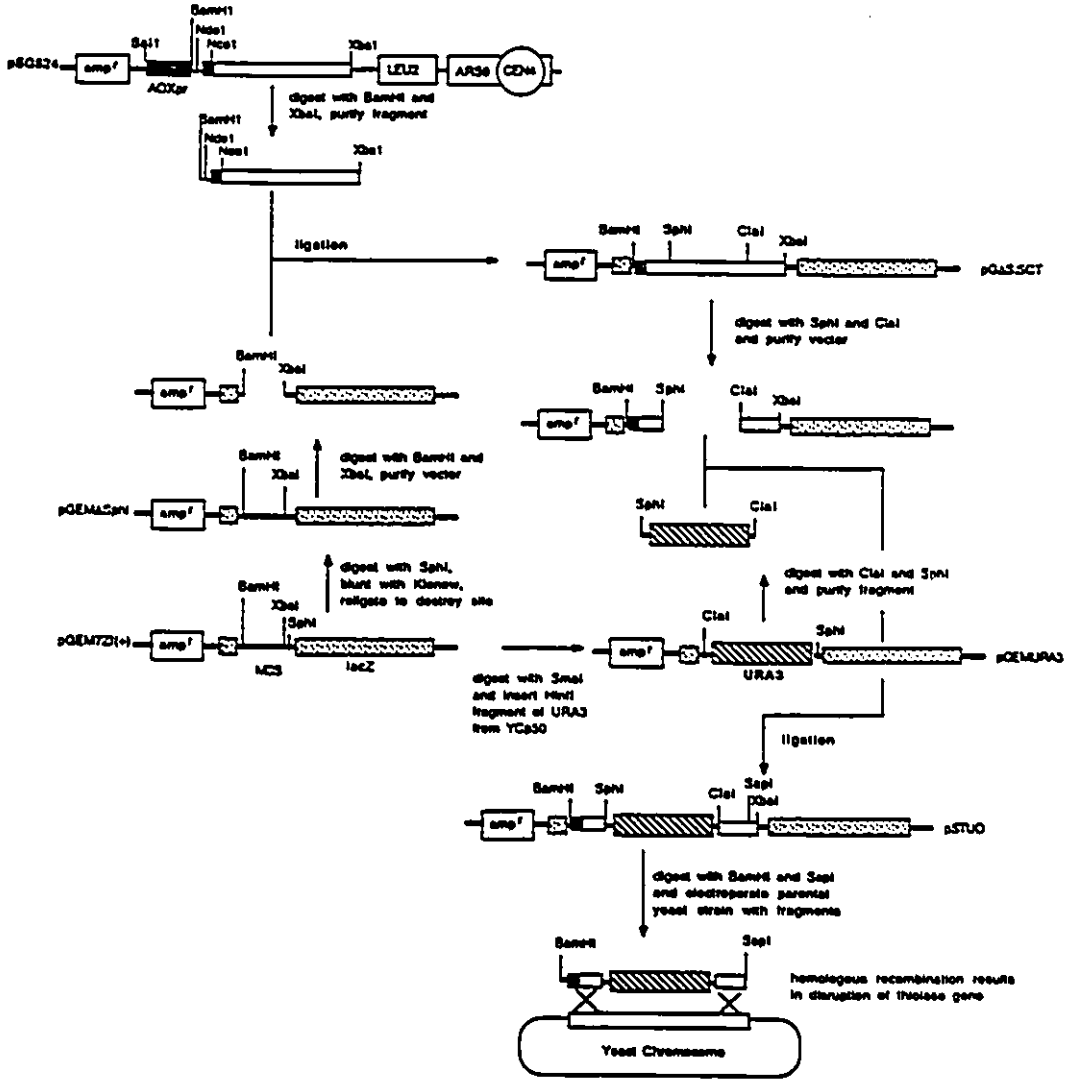

Figure 6. Construction of plasmids used for the disruption of the thiolase gene, *POT1*, by homologous recombination. Details of DNA manipulations are given in the text. pSTUD3 and pSTUD5 have the *URA3* gene inserted into the same sites within the thiolase coding region in opposite orientations.

pGEMURA3-3 and pGEMURA3-5. In order to facilitate insertion of the *URA3* gene into the SCT coding region, a version of pGEM7Zf(+) with the *Sph*I site eliminated from the multiple cloning region was constructed by digestion with *Sph*I, blunting with Klenow fragment, and religation. The resulting plasmid was designated pGEM7Δ*Sph*I. The SCT open reading frame was subcloned as a *Bam*HI/*Xba*I fragment into pGEM7Δ*Sph*I. From this plasmid a *Sph*I and *Cla*I fragment was excised and replaced with *Sph*I/*Cla*I fragments from both pGEMURA3-3 and pGEMURA3-5. The resulting plasmids, pSTUD3 and pSTUD5, were digested with *Bam*HI and *Ssp*I and the resulting fragments were electroporated into DL1.

Table 4. Yeast culture media.

Medium	Composition	Reference
YEPD[a]	2% bacto peptone 1% yeast extract 2% glucose	Rose *et al.*,1988
YNBD[a,b]	0.67% YNB without amino acids 2% glucose	Rose *et al.*, 1988
YNO[a,b]	0.67% YNB without amino acids 0.05% yeast extract 0.5% (w/v) TWEEN 40 0.1% (w/v) oleic acid	Erdmann *et al.*, 1989
SCIM[b]	0.67% YNB without amino acids 0.5% yeast extract 0.5% peptone 0.1% glucose 0.5% (w/v) TWEEN 40 0.1% (w/v) oleic acid	Erdmann *et al.*, 1989

a Solid media made by adding agar to 1.5%. *b* Supplemented with 20 μg·mL^{-1} histidine, 20 μg·mL^{-1} uracil, 20 μg·mL^{-1} lysine, 20 μg·mL^{-1} adenine, and/or 30 μg·mL^{-1} leucine as required.

Uracil prototrophs selected from this transformation were streaked onto YNO plates and incubated at 30°C for several days. Total DNA was prepared from yeasts that failed to grow, digested with *Sau*3AI and subjected to Southern blot analysis. Strains with appropriate patterns of bands on Southern blots were cultured in SCIM and analyzed for expression of thiolase by western blotting.

A similar strategy was used to obtain a thiolase knockout in the yeast strain YPH102 which has a general defect for peroxisome assembly. YPH102 was electroporated with *Bam*HI-*Ssp*I fragments generated from pGEMURA3-3, and colonies capable of growth on minimal media without uracil were selected. Isolates were grown in SCIM and glass bead lysates were assayed for the presence of thiolase by western blotting. YPHSTUD-10, one of the thiolase-deficient isolates, was examined by Southern blotting of total genomic DNA.

2.4.3. TRANSFORMATION OF YEASTS

Introduction of plasmid DNA was accomplished by one of two methods. Yeast was electroporated essentially as described by Becker and Guarente (1991). Cells were grown to mid-log phase in the appropriate medium and washed three times in distilled water and once in 1 M sorbitol. Cells were resuspended in a minimal volume of 1 M sorbitol, and 25 µL of this suspension was mixed with 1 µL of miniprep DNA. A 20 µL aliquot of this mixture was subjected to a brief electrical pulse generating a field of about 7.5 $kV\text{-}cm^{-1}$ in a microelectroporation chamber (BRL). Immediately following

electroporation, the cells were diluted into 100 µL of ice-cold 1 M sorbitol and plated onto selective YNBD agar plates.

Alternatively, when the higher efficiency of electroporation was not required, yeasts were transformed according to Elbe (1992). 1 to 2 µL of miniprep plasmid DNA was mixed together with about 100 µg of sheared, denatured salmon sperm DNA in 250 µL of PLATE (40% PEG 4000, 100 mM lithium acetate, 10 mM Tris-HCl pH 7.5, 1 mM EDTA). A scraping of cells from a streaked plate was transferred into this mixture, vortexed and incubated overnight at room temperature. 50 µL, containing most of the cells which settle overnight, was pipetted from the bottom of the tube and plated onto selective YNBD agar plates.

2.4.4. PREPARATION OF TOTAL CELL LYSATES

Total cell lysates were prepared according to the method of Needleman and Tzagoloff (1975). Cells were vortexed together with glass beads (400 mesh) in a buffer consisting of 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM $ZnCl_2$. 1 mM phenylmethylsulfonyl fluoride was routinely added to the lysis buffer. However some proteins of interest were apparently sensitive to proteolytic activity during lysis. In these cases a cocktail of protease inhibitors including 0.5 $U \cdot mL^{-1}$ aprotinin, 2.5 $\mu g \cdot mL^{-1}$ chymostatin, 2.5 $\mu g \cdot mL^{-1}$ pepstatin, 2.5 $\mu g \cdot mL^{-1}$ antipain, 2.5 $\mu g \cdot mL^{-1}$ leupeptin, 4 $\mu g \cdot mL^{-1}$ TLCK, 2.5 mM NaF, and 1 mM PMSF was added to the buffer. When required, protein concentration was assayed by the dye-binding method of Bradford

(1976) using ovalbumin as a standard and a commercially available dye reagent (BioRad).

2.4.5. SUBCELLULAR FRACTIONATION OF YEAST

Peroxisomes were isolated from *S. cerevisiae* essentially as described in Lewin *et al.* (1990). Unless otherwise indicated operations were carried out at 4°C. Cells cultured in SCIM were collected and washed three times in distilled water at room temperature. The washed cells were resuspended at a density of about 0.5 $g \cdot mL^{-1}$ in zymolyase digestion buffer (0.5 M KCl, 5 mM MOPS pH 7.2, 10 mM Na_2SO_3, and including protease inhibitors) containing either 0.25 $mg \cdot mL^{-1}$ zymolyase 100T or 0.5 $mg \cdot mL^{-1}$ zymolyase 20T and digested for 30 min to 1 h with gentle agitation at 30°C to generate spheroplasts. Spheroplasts were collected by centrifugation at 2,000 x *g* for 8 min and resuspended at 0.5 $g \cdot mL^{-1}$ in disruption buffer (5 mM MES pH 6.0, 0.5 mM EDTA, 0.6 M sorbitol, 0.1% (v/v) ethanol), usually with protease inhibitors present. The cells were homogenized with 10 to 15 strokes of a Potter-Elvehjem homogenizer, and the homogenate was cleared of cell debris and nuclei by centrifugation at 1,000 x *g* for 10 min. This postnuclear supernatant was centrifuged at 20,000 x *g* for 20 min to yield a supernatant enriched for cytosolic proteins and a pellet enriched for organelles.

The pellet was drained and rinsed with disruption buffer and then resuspended in a small volume of disruption buffer as gently as possible. The resuspended pellet was layered onto a discontinuous density gradient consisting of steps of 0.5 mL 50%, 0.7 mL 35%, 2.5 mL 25% and 1.1 mL 17% Nycodenz™ in disruption buffer and centrifuged

in a Beckman VTi65 rotor at 135,000 x *g* for 60 min at 4°C. Gradients were fractionated using a peristaltic pump. Fractions of about 250 to 500 µL were collected. The density of fractions was determined by refractometry or calculated by measuring the mass and volume of each fraction.

For rapid analysis of large numbers of strains which did not require further fractionation of the organellar pellet, a reduced scale procedure was employed. 10 mL cultures were harvested, washed and spheroplasted as above using proportionally smaller volumes of buffers. The spheroplasts were usually resuspended in 0.5 mL of disruption buffer and homogenized in a small tissue grinder (working capacity 2 mL). The postnuclear supernatant was obtained following centrifugation at 1,600 x *g* for 10 min in a 1.5 mL Eppendorf microfuge tube in a HB-4 swinging bucket rotor fitted with adaptors. A 400 µL aliquot of the postnuclear supernatant was layered onto a cushion of 17% Nycodenz and centrifuged at 25,000 x *g* for 20 min in a TLA100.2 rotor. The supernatant was carefully retrieved without disturbing the cushion which was then decanted. The pellet was rinsed with disruption buffer and then resuspended in 400 µL of disruption buffer.

2.4.6. ENZYME ASSAYS

Catalase

Catalase activity was measured essentially as described by Leighton *et al.* (1968). Samples of up to 50 µL in volume were solubilized in 50 µL of 2% Triton X-100 and

incubated on ice-water. 1 mL of substrate solution consisting of 20 mM imidazole buffer pH 7.0, 1 mg·mL^{-1} BSA, and 0.01% H_2O_2 was added and incubated for 5 to 10 min on ice-water. The reaction was terminated by the addition of $TiOSO_4$ in 2N H_2SO_4 and the yellow peroxy-titanium sulfate complex was quantitated by measuring the absorbance at 410 nm.

Cytochrome *c* oxidase

Cytochrome *c* oxidase, a marker enzyme for the mitochondrion, was measured according to Cooperstein and Lazarow (1951). Triton X-100 (250 µL of a 10% solution) was added to 2.5 mL of substrate consisting of 0.38 mg·mL^{-1} cytochrome *c* in 0.3 M ammonium acetate pH 7.4. A few crystals of sodium hydrosulfite (sodium dithionite) were added to reduce the cytochrome *c*. The solution was vigorously aerated by shaking until the cytochrome *c* was oxidized slightly as signalled by a decrease in absorbance at 550 nm. Sample was then added, and the rate of cytochrome *c* oxidation was measured as a decrease in absorbance at 550 nm.

3-Ketoacyl-CoA Thiolase

Thiolase activity was measured essentially as described by Suebert *et al.* (1968), except that the assay was performed using 3-hydroxydecanoyl-CoA (a gift from W.-H. Kunau, Bochum) so that enzymatic conversion of the enoyl-CoA derivative by enoyl-CoA hydratase was circumvented. 3-hydroxydecanoyl-CoA was added to a final concentration of 50 µM to a solution of 50 mM Tris-HCl pH 9.0, 50 mM KCl, 25 mM $MgCl_2$, 50 µg·mL^{-1} BSA, 1 mM NAD, 1 mM sodium pyruvate, 25 mU pig heart 3-hydroxyacyl-

CoA dehydrogenase, 1.8 U rabbit muscle lactate dehydrogenase. The formation of 3-ketodecanoyl-CoA was monitored spectrophotometrically as an increase in absorbance at 303 nm, signalling the formation of a Mg^{2+} complex. Measurement of thiolytic activity was initiated by the addition of sample previously solubilized on ice in 1% Triton X-100 and of CoASH to a final concentration of 150 µM. Activity was calculated using an extinction coefficient of 13.9 $cm^2 \cdot \mu mol^{-1}$ (W.-H. Kunau, personal communication).

2.5. PROTEIN ANALYSIS

2.5.1. ELECTROPHORESIS

For routine analysis of proteins, a 10% discontinuous PAGE system was used (Laemmli, 1970). When required, gels were stained with Coomassie blue and destained to reduce background. For analysis of yeast strains expressing epitope-tagged thiolase which is larger than wild-type thiolase by only 5 amino acid residues, a 7 to 15% gradient resolving gel was employed.

2.5.2. ELECTROPHORETIC TRANSFER

For western blot analysis, gels were transferred to nitrocellulose as described in Burnette (1981). After transfer nitrocellulose blots were blocked with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20) containing 1% powdered skim milk.

2.5.3. PRIMARY DETECTION REAGENTS

Rabbit polyclonal antiserum against *S. cerevisiae* thiolase was a gift from Dr. W.-H. Kunau (Bochum, Germany) and was used at a 1:5000 dilution.

Rabbit polyclonal antiserum against mouse dihydrofolate reductase was from Dr. Suresh Subramani (La Jolla, CA) and was used at a dilution of 1:1000.

Rabbit polyclonal antiserum against *E. coli* chloramphenicol acetyl transferase was a gift from Dr. John P. Capone (Hamilton, ON) and additional sera were raised in this laboratory against a maltose binding protein/chloramphenicol acetyl transferase fusion protein. These sera were used at dilutions of 1:1000.

The 12CA5 monoclonal antibody, which recognizes a 9 amino acid peptide YPYDVPDYA from influenza hemagglutinin, was obtained as an ascites fluid from Berkeley Antibody Company (Richmond, CA) and was used at a dilution of 1:15,000.

The original rabbit polyclonal antiserum (anti-SKL serum) against a synthetic peptide corresponding to the C-terminus of firefly luciferase chemically linked to keyhole limpet hemocyanin (KLH) was a gift from Dr. S. Subramani (La Jolla, CA). An additional serum was raised in this laboratory using an identical strategy as was employed to make the original antiserum (Gould *et al.*, 1990a). Anti-SKL serum has been shown to detect several proteins known to terminate in SKL and several proteins of unknown sequence which are also peroxisomal and probably end in SKL (Gould *et al.*, 1990a; Aitchison *et al.*, 1992). Both sera were used at a dilution of 1:200.

After an incubation of not less than 1 h with primary antibody diluted in TBST,

the blots were subjected to 3 X 10 min washes with TBST.

2.5.4. SECONDARY DETECTION REAGENTS AND DETECTION METHODS

Three methods were employed to visualize proteins detected by primary antibodies used for Western blotting.

Enhanced Chemiluminescence (ECL)

Probed membranes were treated with either a horse radish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (Amersham) diluted 1:30,000 or HRP-conjugated donkey anti-mouse IgG (Amersham) diluted 1:10,000. After washing, the nitrocellulose was blotted on Whatman 3MM paper to remove excess buffer and treated with a commercially available reagent (Enhanced Chemiluminescence or ECL™, Amersham). Light given off by the reaction of H_2O_2 and luminol catalyzed by HRP was detected by exposing the blot to Kodak XOMAT-AR X-ray film for 1 sec to 60 min.

Alkaline Phosphatase

Nitrocellulose membranes that had been probed with primary antisera were washed and probed with either alkaline phosphatase (AP)-conjugated-anti rabbit IgG(Fc) (Promega) diluted 1:75,000 or AP-conjugated anti-mouse IgG(H+L) (Promega) diluted 1:10,000. The nitrocellulose was washed three times in TBST and once with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The blots were then incubated in AP buffer with a 1:1 molar ratio of nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP). When colour was deemed to have developed

adequately the blots were transferred to TE pH 8.0 to stop the reaction.

^{125}I-Protein A

For quantitation by densitometry or by phosphor imager technology, blots were probed with ^{125}I-Protein A (30 mCi·mg^{-1}; Amersham) at a concentration of 1 μCi·mL^{-1}. The blots were washed and exposed either to X-ray film (XOMAT-AR; Kodak) or to a phosphor storage screen (Molecular Dynamics).

Detection of yeast-expressed Protein A polypeptides on nitrocellulose blots

The detection of Protein A-based fusion proteins was a special case in that primary antibody probes were not required because of the ability of Protein A on nitrocellulose blots to bind IgG directly. Two reagents were used for this purpose. A non-specific rabbit IgG fraction conjugated to AP (a gift from Dr. D.W. Andrews) was used at a dilution of 1:200. Reactive bands were visualized as described above for other AP-conjugated secondary antibodies. A second reagent was produced by radioiodination of Protein A-purified rabbit IgG derived from preimmune rabbit serum. The final product had a specific activity of 2.5 mCi·mg^{-1} and was used at a concentration of 5 μCi·mL^{-1}. Probed and washed blots were either exposed to X-ray film or to a phosphor storage screen.

2.5.5. *IN VITRO* TRANSCRIPTION AND TRANSLATION

The *Bam*HI/*Xba*I fragment containing the coding region of SCT was subcloned between the *Bam*HI and *Xba*I sites in the plasmid pGEM7Zf(+) (Promega). Plasmid

DNA was prepared by alkaline lysis followed by centrifugation through cesium chloride (Maniatis *et al.*, 1982) in order to avoid carrying over any RNase. RNA was transcribed in the presence of cap analogue (NEB) using SP6 RNA polymerase and reagents supplied in a kit (Promega) according to the manufacturer's recommendations. *In vitro* transcribed RNA was translated in nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-methionine. Details of the method employed for simultaneous detection of ^{35}S-labelled and immunoreactive proteins is given in the legend of Fig. 17.

2.5.6. CELL LABELLING AND IMMUNOPRECIPITATION

Start-up cultures were grown in YNBD overnight. The cells were harvested by centrifugation, washed with sterile water, transferred to YNO and induced for 6 h. About 3 to 5·10^8 cells were washed with water, transferred to 1 mL YNO with 100 µCi of ^{35}S-methionine·10^8 cells and labelled for 15 min at 30°C. The labelled cells were washed once with YNO containing 20 mM unlabelled methionine and incubated for a further 20 min at 30°C in this medium. The cells were processed as described previously (Section 2.4.5) for reduced-scale fractionation experiments with a cocktail of protease inhibitors and 2 mM unlabelled methionine present in all buffers.

The 20,000 x *g* supernatant was brought to 10 mM Tris-HCl pH 8.5, 2.5 mM EDTA 500 mM NaCl (solubilization buffer) and incubated for 30 min on ice. The solubilized fractions were centrifuged at 100,000 X *g* for 20 min at 4°C. The supernatants were split into equal aliquots, mixed with either 2.5 µL of anti-thiolase

serum or 10 μL of 12CA5 ascites fluid and mixed gently for 1 h at room temperature. The immune complexes were adsorbed with fixed *Staphylococcus aureus* cells (Pansorbin™; Calbiochem) with gentle agitation at room temperature for 1 h. The complexes were pelleted by centrifugation and washed 4 times in solubilization buffer. The final pellet was resuspended in 100 μL of SDS-PAGE sample buffer and boiled for 5 min. The immunoprecipitated proteins were run out on SDS-PAGE. The gels were dried onto blotting paper and exposed to a phosphor storage screen.

Table 5. Yeast strains

Strain[a]	Chromosomal genotype	Plasmid name	Plasmid genotype
DL1	*leu2*, *ura3*, *his3*, *POT1*	no plasmid	no plasmid
DL1[DHFR]	*leu2*, *ura3*, *his3*, *POT1*	pDHFRN.AOX	*CEN4*, *ARS6*,*LEU2*,DHFR
DL1[DHFRSCTN]	*leu2*, *ura3*, *his3*, *POT1*	pDHFRSCTN.AOX	*CEN4*, *ARS6*, *LEU2*,DHFR+SCTN
DL1[CAT]^{hc}	*leu2*, *ura3*, *his3*, *POT1*	CAT.HDE.YEp13	2 μm, *LEU2*, CAT
DL1[CAT]	*leu2*, *ura3*, *his3*, *POT1*	CAT.HDE.YCp50	*CEN*/*ARS*, *URA3*, CAT
DL1[CATΔN]	*leu2*, *ura3*, *his3*, *POT1*	pCATΔN.AOX	*CEN4*, *ARS6*, *LEU2*, CATΔ1-38
DL1[CATSCTN]	*leu2*, *ura3*, *his3*, *POT1*	pCATSCTN.AOX	*CEN4*, *ARS6*, *LEU2*, CATΔ1-38+SCT1-16
DL1[gGPrASTOP]	*leu2*, *ura3*, *his3*, *POT1*	pgGPrASTOP.AOX	*CEN4*, *ARS6*, *LEU2*, gGPrASTOP
DL1[gGPrASKL]	*leu2*, *ura3*, *his3*, *POT1*	pgGPrASKL.AOX	*CEN4*, *ARS6*, *LEU2*, gGPrASKL
DL1[gGPrAAKI]	*leu2*, *ura3*, *his3*, *POT1*	pgGPrAAKI.AOX	*CEN4*, *ARS6*, *LEU2*, gGPrAAKI
DL1[gGPrASCTN]	*leu2*, *ura3*, *his3*, *POT1*	pgGPrASCTN.AOX	*CEN4*, *ARS6*, *LEU2*, gGPrASCT1-16
DL1[PrASTOP]	*leu2*, *ura3*, *his3*, *POT1*	pPrASTOP.AOX	*CEN4*, *ARS6*, *LEU2*, PrASTOP
DL1[PrASKL]	*leu2*, *ura3*, *his3*, *POT1*	pPrASKL.AOX	*CEN4*, *ARS6*, *LEU2*, PrASKL
DL1[PrAAKI]	*leu2*, *ura3*, *his3*, *POT1*	pPrAAKI.AOX	*CEN4*, *ARS6*, *LEU2*, PrAAKI
DL1[PrASCTN]	*leu2*, *ura3*, *his3*, *POT1*	pPrASCTN.AOX	*CEN4*, *ARS6*, *LEU2*, PrASCT1-161
DL1[ΔN]	*leu2*, *ura3*, *his3*, *POT1*	pSCTΔN	*CEN4*, *ARS6*, *LEU2*, SCTΔ1-16
DL1[HA]	*leu2*, *ura3*, *his3*, *POT1*	pSCTHA	*CEN4*, *ARS6*, *LEU2*, SCT(HA)2
DL1[ΔN+HA]	*leu2*, *ura3*, *his3*, *POT1*	pSCTΔN/p313SCTHA2	*CEN4*, *ARS6*, *LEU2*, SCTΔ1-16/ *CEN4*,*ARS6*, *HIS3*, SCT(HA)2
DL1[ΔN^{hc}+HA]	*leu2*, *ura3*, *his3*, *POT1*	p425SCTΔN/pSCTHA2	2 μm, *LEU2*, SCTΔ1-16/ *CEN4*, *ARS6*, *HIS3*, SCT(HA)2
DL1[ΔN+HA]^{hc}	*leu2*, *ura3*, *his3*, *POT1*	p425SCTΔN/ p423SCTHA2	2 μm, *LEU2*, SCTΔ1-16/ 2 μm, *HIS3*, SCT(HA)2
STUD	*leu2*, *ura3*, *his3*, *pot1*::URA3	no plasmid	no plasmid
STUD[524]	*leu2*, *ura3*, *his3*, *pot1*::URA3	pSG524	*CEN4*, *ARS6*, *LEU2*, SCT
STUD[522]	*leu2*, *ura3*, *his3*, *pot1*::URA3	pSG522	*CEN4*, *ARS6*, *LEU2*, SCTS2A
STUD[ΔN]	*leu2*, *ura3*, *his3*, *pot1*::URA3	pSCTΔN	*CEN4*, *ARS6*, *LEU2*, SCTΔ1-16
STUD[HA]	*leu2*, *ura3*, *his3*, *pot1*::URA3	pSCTHA2	*CEN4*, *ARS6*, *LEU2*, SCT(HA)2

Strain[a]	Chromosomal genotype	Plasmid name	Plasmid genotype
CY21	*MATα*, *ura*, *leu*	no plasmid	no plasmid
CY22	*MATa*, *ura3-52*, *his*, *trp*	no plasmid	no plasmid
BF305-15d	*MATa*, *ura*, *leu*, *his*, *ade*, *met*, *arg*, *trp*	no plasmid	no plasmid
699	*MATa*, *ura*, *leu*, *his*, *ade* *trp*, *can1-100*	no plasmid	no plasmid
JB516	*ura*, *ade*, *leu*, *his*	no plasmid	no plasmid
YPH1(YNN214)	*ura3-52*, *lys2-801*, *ade2-101*, *pas8*	no plasmid	no plasmid
YPH102	*ura3-52*, *lys2-801*, *ade2-101*, *leu2-Δ1*, *his3-Δ200*, *pas8*	no plasmid	no plasmid
YPHSTUD	*ura3-52*, *lys2-801*, *ade2-101* *leu2-Δ1*, *pas8*, *pot1::URA3*	no plasmid	no plasmid
YPHSTUD[H11Y]	"	pSCTQ6R/H11Y	*CEN4*, *ARS6*, *LEU2*, SCTQ6R/H11Y
YPHSTUD[H11Y/HA]	"	pSCTQ6R/H11Y+ p313SCTHA2	*CEN4*, *ARS6*, *HIS3*, SCT(HA)2

Strain DL1 (van Loon *et al.*, 1983) and plasmids pSG524 and pSG522 were gifts from Dr. Suresh Subramani, University of California, San Diego. CY21, CY22, BF305-15d, 699, and JB516 were all provided by Ms. Debbie Inglis, McMaster University. YPH1 and YPH102 (Sikorski and Hieter, 1989) and plasmids pRS313, pRS315 (Sikorski and Hieter, 1989), pRS423, and pRS425 (Christianson *et al.*, 1992) were gifts from Dr. Liam Good, University of Guelph. All constructs were regluated by a 450 bp promoter of *S. cerevisiae* acyl-CoA oxidase (*POX1*) gene (Dmochowska *et al.*, 1990; Wang *et al.*, 1992) except CAT·HDE·YEp13 and CAT·HDE·YCp50 which are regulated by a 750 bp promoter of *C. tropicalis* trifunctional enzyme (HDE) gene (Aitchison, 1992). Most plasmids were constructed using pRS315 as a backbone, except for those with "313", "423", or "425" in their designations and which were constructed in the corresponding pRS vectors.

3. RESULTS AND DISCUSSION

3.1. THE N-TERMINAL 16 AMINO ACIDS OF *S. CEREVISIAE* ARE SUFFICIENT TO DIRECT A CYTOSOLIC PASSENGER DOMAIN TO PEROXISOMES.

3.1.1. OVERVIEW

In the absence of any specific targeting information that might direct a protein to another location (compartment or membrane), a protein is retained in the cytosol where it is synthesized. There are many examples in which the deletion or disruption of the targeting information results in the modified protein remaining cytosolic while, conversely, addition of a complete targeting signal to a cytosolic protein results in the correct targeting of the "passenger" protein (Pugsley, 1990). This straightforward concept leads directly to two related experimental endeavors with respect to thiolase targeting. In order to demonstrate that the N-terminus of thiolase is *necessary* for peroxisomal targeting, a truncated version must be constructed and remain cytosolic when expressed in yeast. To prove that the deleted region contains *sufficient* information for targeting, it must be fused to a cytosolic passenger protein and be able redirect that protein to peroxisomes. When applied to the N-terminus of *S. cerevisiae* thiolase, neither experiment proved to be as straight forward as one might expect. Initial results of the first of the two experiments seemed to indicate that deletion of the

N-terminal 16 amino acids of thiolase only reduced the efficiency of targeting and did not abolish it completely (see section 3.3). This observation was contrary to my conviction that the region contained residues corresponding to the minimal PTS2 of rat thiolase as determined by Swinkels *et al.* (1991). At an impass, I decided to address the question of sufficiency of the thiolase N-terminus in the targeting of a passenger protein. Two of three passenger domains that were fused to the N-terminus of thiolase proved to be inherently uninformative while the third "worked" well.

3.1.2. DHFR PASSENGER DOMAIN

Mouse dihydrofolate reductase (DHFR) might be considered an ideal candidate for serving as a passenger domain in that it has low molecular weight, is monomeric, and has been well characterized in this capacity both *in vivo* and *in vitro*. The protein has been fused to both endoplasmic reticulum and mitochondrial targeting signals and has been shown to be targeted faithfully to these compartments. Particularly relevant to this work the fact that DHFR had been successfully used as a passenger protein and targeted to peroxisomes by the N-terminal PTS2 of rat liver peroxisomal thiolase in transfected CHO cells (Osumi *et al.*, 1991). DHFR is also ideal for the examination of the role of protein structure in import. Binding of the folate analogue methotrexate stabilizes the folded structure of DHFR and abolishes import (Eiders and Schatz, 1986; Eiders *et al.*, 1988). These are among several key experiments that shape the widely held notion that proteins must unfold for translocation across membranes.

A drawback to the use of DHFR for fusion to a peroxisomal targeting signal is the observation that DHFR with the SKL-COOH tripeptide fused to the C-terminus of the protein was unable to be imported *in vivo* in yeast (Distel *et al.*, 1992; Kragler *et al.*, 1993). The observation that fusions between DHFR and 6 or more amino acids of the luciferase C-terminus could be imported into peroxisomes possibly indicates that the tripeptide alone may be masked in the DHFR fusion protein and that a "spacer" of additional amino acids sufficient to expose the targeting signal to the recognition and/or translocation apparatus may be required.

The unfused DHFR passenger domain used in this experiment was modified by the cloning strategy used so that the predicted N-terminal amino acid sequence was Met-Ala-Gly-Pro... *vs.* Met-Val-Arg-Pro... found at the N-terminus of the protein encoded by the unmodified cDNA. The DHFR-SCTN construct was made by fusion of the codon for Met17 of thiolase to the codon for the initiation codon of the modified DHFR domain (see Fig. 3).

Subcellular fractionation indicated that both proteins were cytosolic (Fig. 7). DHFR was not targeted by an N-terminal sequence containing the putative PTS2 motif in *in vivo* experiments in yeast.

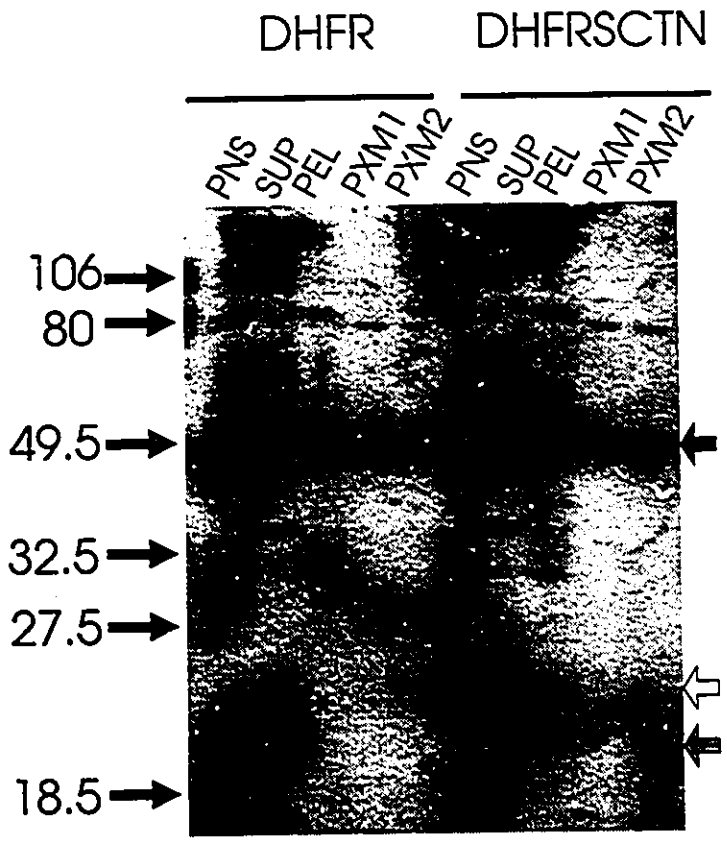

Figure 7. Western blot analysis of subcellular fractions of yeast strains expressing DHFR constructs. 500 mL cultures of DL1[DHFR] and DL1[DHFRSCTN] were grown for 20 h in SCIM. The peroxisome fraction from the first Nycodenz™ gradient (PXM1) was diluted in disruption buffer and re-isolated on a second gradient (PXM2). Samples of the post-nuclear supernatant (PNS), 20,000 x *g* supernatant (SUP) and 20,000 x *g* pellet (PEL) representing equivalent portions of each fraction as well as 10 µg of peroxisomal protein, were resolved by SDS-PAGE on a 10% gel. The proteins were transferred to nitrocellulose and probed with anti-*S. cerevisiae* thiolase and anti-mouse DHFR sera followed by ^{125}I-Protein A. The blot was exposed to X-ray film at -70°C for 48 h with an intensifying screen. Filled arrow = thiolase. Open arrow = DHFRSCTN. Grey arrow = DHFR. A DHFR cross-reactive band is marked with an asterisk. M_r of BioRad prestained markers is given in kDa.

3.1.3. CAT PASSENGER DOMAIN

E. coli chloramphenicol acetyl transferase (CAT) was considered to be a good candidate for a passenger protein primarily because it has been used to demonstrate peroxisomal targeting in mammalian cells fused to both a C-terminal SKL-COOH (Gould *et al.*, 1989) and the N-terminal PTS2 of rat thiolase (Swinkels *et al.*, 1991).

A drawback with respect to the utility of this protein was the observation that CAT fused to the C-terminal tripeptide of *C. tropicalis* trifunctional enzyme (HDE) was not targeted to peroxisomes in *S. cerevisiae* even though this tripeptide was shown to be necessary for targeting of HDE (Aitchison *et al.*, 1991; Aitchison, 1992). The oligomerization of CAT was thought to impede the import of CAT polypeptides into yeast mitochondria *in vivo* (Nye and Scarpulla, 1990). A deletion in the CAT domain required for tetramerization resulted in efficient import into mitochondria.

The strategy used to fuse the *S. cerevisiae* thiolase PTS2 to CAT resulted in the deletion of a substantial portion of the N-terminus of CAT (see Fig. 4). A possible benefit of this particular strategy is that it reduces the chance that the remaining portion of CAT can fold into an oligomeric structure or into a structure which restricts the interaction between the targeting signal and the import machinery. Analysis of the expression of full-length CAT, CATSCTN, and CATΔN indicated that the truncated and fused proteins were synthesized at their expected M_r and at levels comparable to that of the full-length protein (Fig. 8).

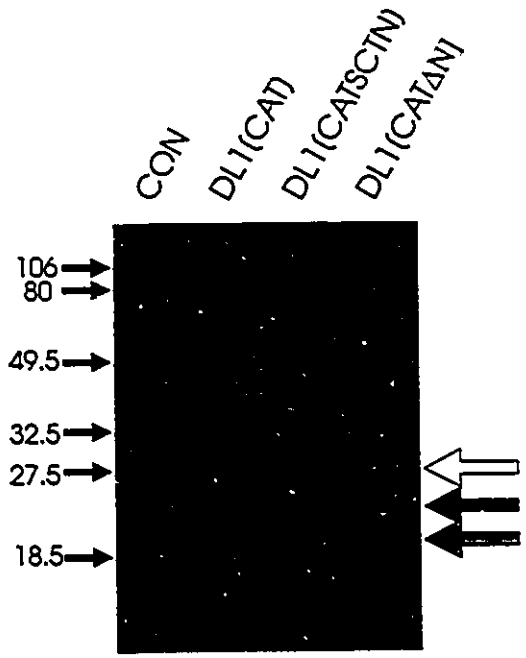

Figure 8. Western blot analysis of expression of CAT constructs. 10 mL cultures of DL1[CAT], DL1[CATΔN], and DL1[CATSCTN] were grown in SCIM for 18 h. Equal amounts of glass bead lysates were resolved by SDS-PAGE on a 10% gel. Proteins were transferred to nitrocellulose which was probed with anti-CAT serum followed by HRP-conjugated anti-rabbit IgG and detected by ECL™ with a 10 s exposure. Filled arrow = full-length CAT. Open arrow = CATSCTN. Grey arrow = truncated CAT (CATΔN). M_r of BioRad prestained markers is given in kDa.

A detailed analysis of the subcellular localization of CAT polypetides in induced cultures showed unexpectedly that the unfused truncated protein comigrated with the peroxisomal fraction as did the CAT domain fused to the thiolase N-terminus (Fig. 9). The nature of the apparent colocalization of CATΔN and thiolase was not precisely determined (imported *vs.* adherence to the peroxisomal membrane) although the two proteins comigrated with peroxisomes on a second Nycodenz™ gradient and were equally protected from protease digestion in the absence of detergents (not shown). Inspection of the sequences "exposed" by the N-terminal truncation of CAT did not reveal an obvious cryptic peroxisomal targeting signal. It may also be possible that the expression of the truncated form of CAT resulted in the formation of protein aggregates with a density similar to that of peroxisomes as was observed for various truncated forms of catalase expressed at high levels from multi-copy vectors (Hartig *et al.*, 1990).

In earlier work I had observed that full-length CAT was partially particulate when expressed from a high-copy number plasmid vector. This sedimentable form of CAT comigrated with mitochondria (Fig. 10, panel A). As indicated by immunoblotting of Nycodenz™ density gradient fractions with antiserum specific for cytochrome *c* oxidase subunit II, the distribution of CAT with mitochondrial protein was precise (Fig. 10, panel B). When expressed on a low copy-number plasmid (YCp50) the level of CAT protein was about 10-fold lower than when expressed from the high copy-number vector YEp13 (Fig. 10, panel C) and was located entirely in the cytosolic fraction (Fig. 10, panel D).

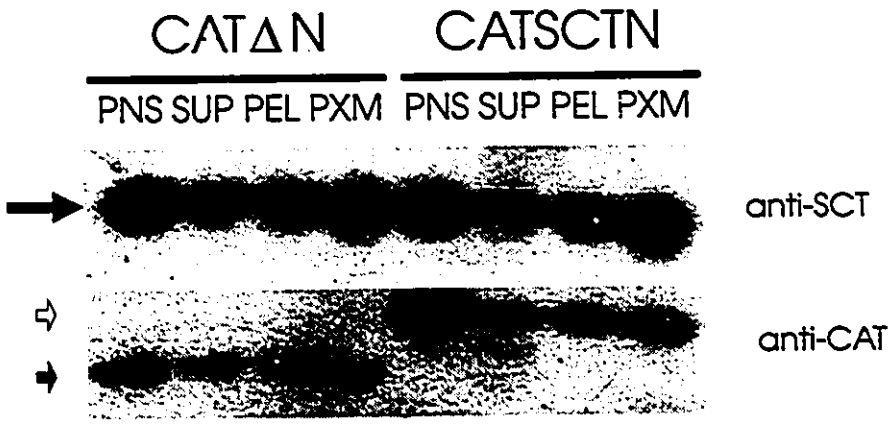

Figure 9. Western blot analysis of subcellular fractions of yeast strains expressing CAT constructs. 500 mL cultures of DL1[CATΔN] and DL1[CATSCTN] were grown for 18 h in SCIM. Samples of the post-nuclear supernatant (PNS), 20,000 x *g* supernatant (SUP) and 20,000 x *g* pellet (PEL), representing equivalent portions of each fraction, as well as 10 μg of peroxisomal protein (PXM) and 10 μg of mitochondrial protein (MIT), were resolved by SDS-PAGE on a 12.5% gel. Proteins were transferred to nitrocellulose which was probed with anti-CAT and anti-*S. cerevisiae* thiolase sera followed by ^{125}I-Protein A. The western blot was exposed to X-ray film at -70°C for 48 h with an intensifying screen. Filled arrow = thiolase. Filled arrowhead = CATΔN. Open arrowhead = CATSCTN. M_r of BioRad prestained markers is given in kDa.

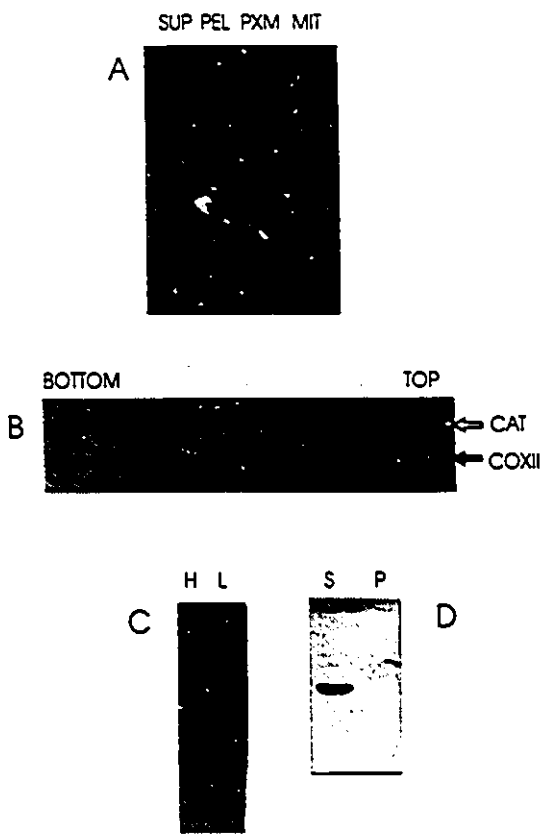

Figure 10. Western blot analysis of subcellular fractions of yeast strains overexpressing full-length CAT. Panels A and B: A 500 mL culture of DL1[CAT]^{hc} was fractionated into 20,000 x *g* supernatant and pellet. The pellet was further fractionated on a discontinuous Nycodenz™ density gradient. Panel A. Equal portions of 20,000 x *g* supernatant (SUP, lane 1) and 20,000 x *g* pellet (PEL, lane 2), as well as 10 μg of peroxisomal (PXM, lane 3) and mitochodrial (MIT, lane 4) protein were resolved by SDS-PAGE on a 10 % gel and proteins were transferred to nitrocellulose. Blots were probed with anti-CAT serum followed by HRP-conjugated anti-rabbit IgG. Immunoreactive bands were visualized by ECL™. Panel B: Equal portions of gradient fractions were resolved by SDS-PAGE and the nitrocellulose blot was probed with anti-CAT and anti-COXII sera. Panel C: Equal amounts of glass bead lysate prepared from 10 mL cultures of DL1[CAT]^{hc} (H) and DL1[CAT] (L), and the level of expression of CAT was assessed by western blotting as in panel A. Panel D: Equal amounts of 20,000 x *g* supernatant (S) and 20,000 x *g* pellet (P) were subjected to western blot analysis as in panel A.

The experiments with DHFR and CAT illustrate the perils of heterologous protein expression in yeast and the unpredictability of each new experimental endeavour.

3.1.4. GLYCOGLOBIN/PROTEIN A PASSENGER DOMAIN

A further opportunity to investigate peroxisomal targeting of passenger protein domains in yeast arose as a result of a collaboration with Dr. F. Janiak and Dr. D.W. Andrews from the Department of Biochemistry, McMaster University. These investigators were primarily concerned with identifying a passenger domain which would respond correctly to a variety of fused signals while remaining passive with respect to the property being examined if used in the unfused form.

Protein A of *S. aureus* is an IgG-binding protein normally located anchored in the outer membrane of the organism with the IgG-binding domain facing the extracellular medium. An altered gene encoding a cytosolic version of Protein A lacking the bacterial secretory signal and the membrane anchoring domain but retaining the IgG-binding domains formed the bulk of the passenger protein. Unexpectedly, a fusion between the signal sequence of preprolactin and the IgG-binding domain of Protein A (PrA) was not translocated into the lumen of endoplasmic reticulum vesicles in *in vitro* import experiments due to the absence of SRP-signal interaction (Janiak *et al.*, 1994). Because the sequence of amino acids located within a short distance from the cleavage site hade been shown to influence co-translational import in this type of assay (Andrews *et al.*, 1988) a spacer composed of the N-terminal 27 amino acids of a modified

chimpanzee α-globin was inserted between the signal and the PrA domain. The preprolactin signal fused to the modified passenger (gGPrA) was subsequently recognized by SRP and the modified passenger domain was correctly translocated in to the endoplasmic reticulum lumen (Janiak *et al.*, 1994).

The goal of our collaboration was to explore the potential of gGPrA in *in vivo* targeting experiments in yeast. Synthetic oligonucleotides were inserted downstream of the Protein A IgG-binding domain coding region (see Fig. 5) resulting in recombinant genes encoding at their C-termini the amino acid sequences ...Ile-Leu-COOH (gGPrASTOP), ...Ile-Leu-Ala-Lys-Ile-COOH (gGPrAAKI), and ...Ile-Leu-Ser-Lys-Leu-COOH (gGPrASKL).

Analysis of crude glass bead lysates of induced cultures revealed that proteins of the expected M_r were present (Fig. 11). However in a crude fractionation procedure only the gGPrA proteins fused to PTS1 tripeptides were detected in the organellar pellet and in the fractions enriched for peroxisomes (Fig. 12, panels B and C). The protein expressed from the gGPrASTOP construct was difficult to detect or substantially degraded unless a cocktail of protease inhibitors was included throughout the fractionation procedure. When detected, it was found in the cytosolic fraction (Fig. 12, panel A). A simple interpretation of this result is that endogenous proteases released during the fractionation procedure have access to and can degrade the cytosolic form of gGPrA in contrast to the PTS1-fused proteins which are protected from degradation by the peroxisomal membrane. The cytosolic protein was detected in lysates because

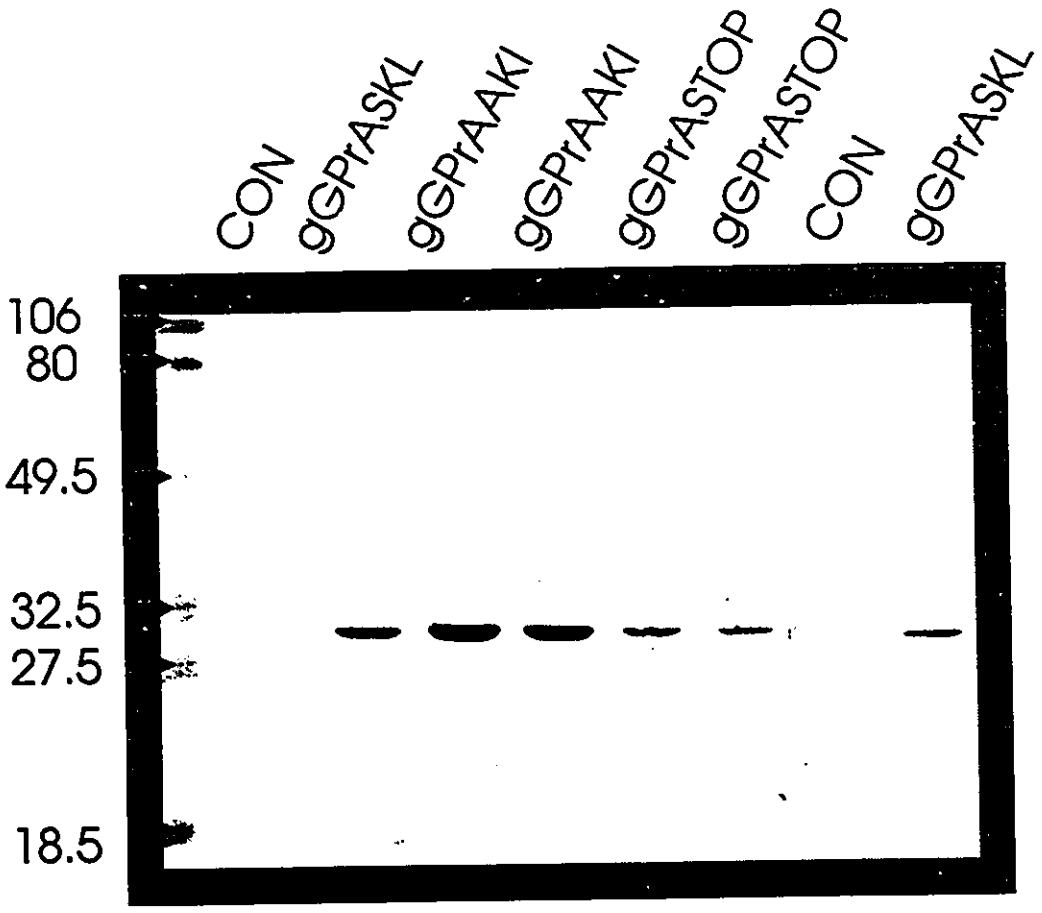

Figure 11. Western blot analysis of the expression of gGPrA. 10 mL cultures of DL1 (CON), DL1[gGPrASTOP], DL1[gGPrAAKI] and DL1[gGPrASKL] were grown in SCIM for 18 h. Equal amounts of glass bead lysates were resolved by SDS-PAGE on a 12% gel. Proteins were transferred to nitrocellulose and the blot was probed with non-specific AP-conjugated rabbit IgG and detected with NBT/BCIP. M_r of BioRad prestained molecular weight markers is given in kDa.

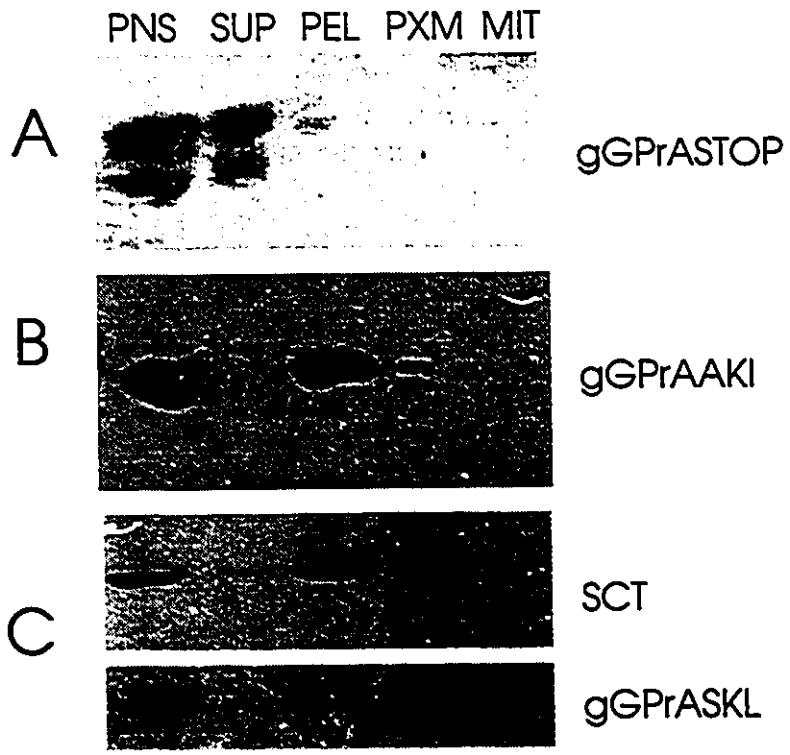

Figure 12. Western blot analysis of subcellular fractions of yeast expressing gGPrA constructs. 500 mL cultures of DL1[gGPrASTOP], DL1[gGPrAAKI], and DL1[gGPrASKL] were grown for 20 h in SCIM. Equivalent proportions of the post nuclear supernatant (PNS), 20,000 x *g* supernatant (SUP) and 20,000 x *g* pellet (PEL), along with 5 µg each of peroxisomal (PXM) and mitochondrial (MIT) protein from density gradient fractions, were resolved by SDS-PAGE on a 10% gel. The proteins were transferred onto nitrocellulose. Panels A and B: Blots were probed with non-specifc AP-conjugated rabbit IgG and developed with NBT/BCIP. Panel C: Blot was divided into upper and lower segments. The upper segment was probed with anti-*S. cerevisiae* thiolase serum followed by ^{125}I-Protein A. The lower segment was probed with non-specific iodinated IgG purified from pre-immune rabbit serum. The blots were exposed to X-ray film at -70°C with an intensifying screen.

the procedure required to obtain lysates is very brief in comparison to that required to homogenize and fractionate cells.

A more detailed examination of the subcellular location of gGPrASKL indicated that it co-fractionates exactly with endogenous thiolase on Nycodenz gradients (Fig. 13). This was equally true for gGPrAAKI (not shown) demonstrating for the first time that these targeting signals are sufficient for peroxisomal targeting in *S. cerevisiae*.

The success of the experiments showing targeting of the gGPrA domain to peroxisomes *via* the PTS1 pathway led directly to an experiment to determine if gGPrA could also be routed to peroxisomes by the putative PTS2 in the *S. cerevisiae* thiolase N-terminus. The codon encoding Met17 of *S. cerevisiae* thiolase was fused to the codon encoding the initiation Met of gGPrASTOP (see Fig. 5 for construction of gGPrASCTN.ACT). Expression in yeast resulted in the synthesis of a protein of the expected M_r which was colocalized with thiolase on fractionation (Fig. 14).

To ascertain whether gGPrA fusions were localized inside peroxisomes as opposed to forming inclusion bodies with similar sedimentation properties or inserted into the peroxisomal membrane, peroxisomes purified on Nycodenz™ gradients from DL1[gGPrASKL] and DL1[gGPrASCTN]. Treatment with Triton X-100 was expected to solubilize the peroxisomal membrane and release soluble matrix elements whereas inclusion bodies would remain in a sedimentable particulate fraction. Treatment with SDS was expected to solubilize even inclusion bodies. Treatment with Tris-HCl pH 8.5 causes the peroxisomal membrane to be removed intact and any proteins associated with

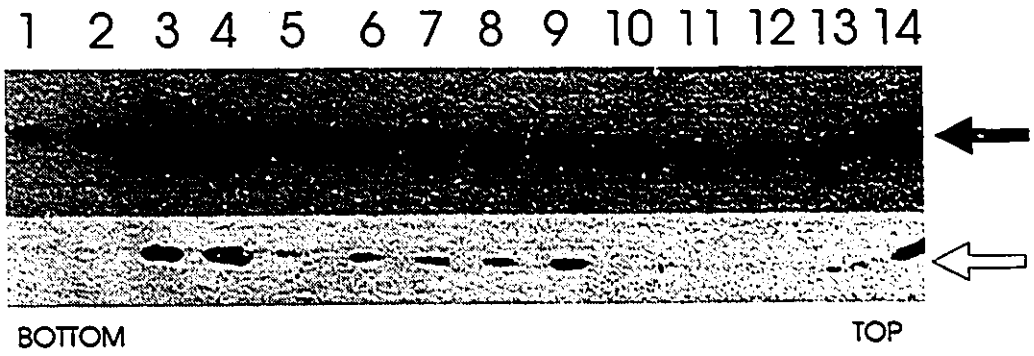

Figure 13. Western blot analysis of organellar pellet from yeast expressing gGPrASKL fractionated on Nycondenz™ density gradients. The organellar pellet prepared from a 500 mL culture of DL1[gGPrASKL] grown for 18 h in SCIM was resuspended and organelles separated on a discontinuous Nycodenz™ density gradient. An equal portion of each fraction was resolved by SDS-PAGE on a 10% gel. The proteins were transferred to nitrocellulose and the blot was divided into upper and lower portions. The upper portion was probed with anti-*S. cerevisiae* thiolase serum followed by HRP-conjugated anti-rabbit IgG and detected with ECL™ with a 10 s exposure. The lower panel was probed with nonspecific AP-conjugated rabbit IgG and detected with NBT/BCIP. Filled arrow = thiolase. Open arrow = gGPrASKL.

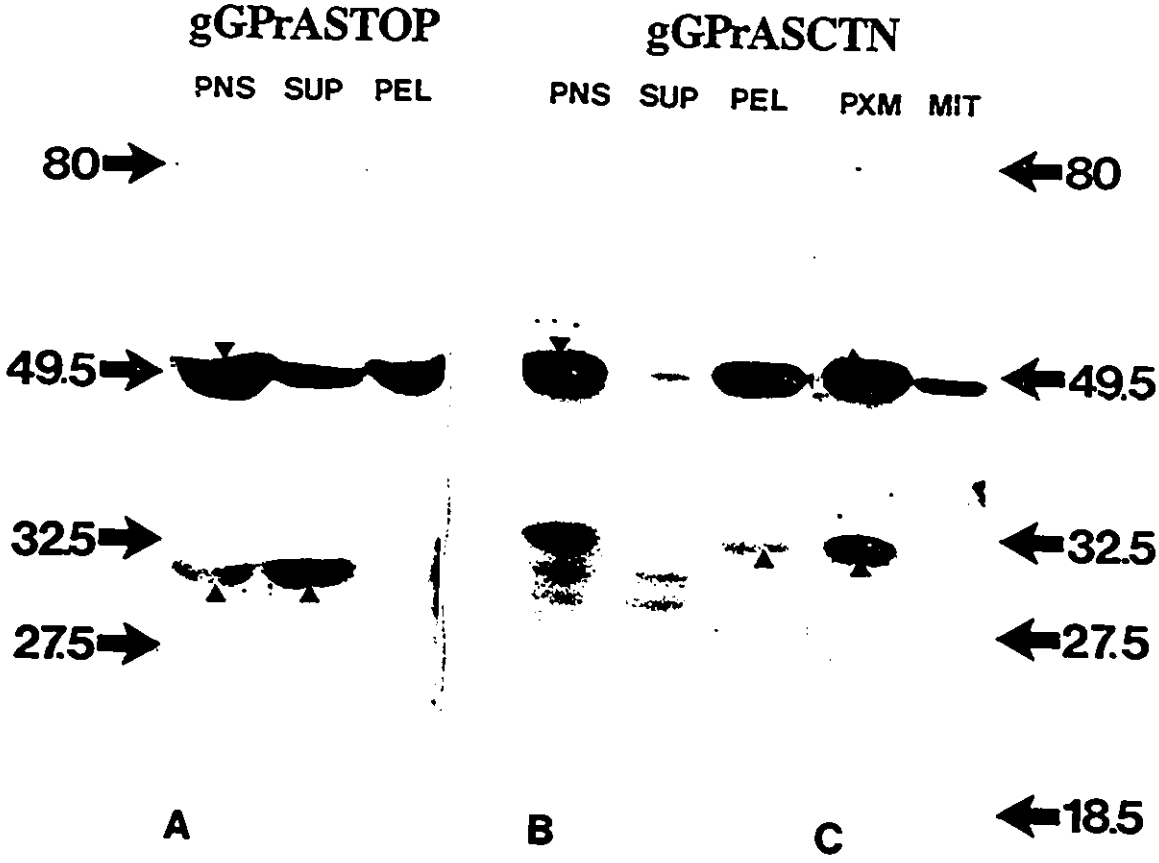

Figure 14. Western blot analysis of subcellular fractions of yeast strain expressing gGPrA fused to the thiolase N-terminus. 500 mL cultures of DL1[gGPrASTOP] and DL1[gGPrASCTN] were grown for 18 h in SCIM. Equivalent proportions of the postnuclear supernatants (PNS), 20,000 x *g* supernatants (SUP) and 20,000 x *g* pellets (PEL), as well as 5 μg of peroxisomal (PXM) and mitochondrial (MIT) protein from density gradient fractionation of the pellet from DL1[gGPrASCTN] cells, were resolved by SDS-PAGE on a 10% gel. The proteins were transferred to nitrocellulose and the blot was divided into upper and lower segments. The upper segment was probed with anti-*S. cerevisiae* thiolase serum followed by ^{125}I-Protein A. The lower panel was probed with pre-immune iodinated rabbit IgG. The blots were exposed to X-ray film at -70°C. Downward pointing arrowheads = thiolase. Upward pointing arrowheads = gGPrA and gGPrASCTN. M_r of BioRad prestained molecular weight markers is given in kDa.

it should sediment during centrifugation. Treatment with carbonate should perform a similar action and solubilize proteins that are peripherally associated with the peroxisomal membrane. The results of these treatments are shown in Fig. 15.

Triton X-100 treatment released little thiolase over and above that observed in the untreated peroxisomes, possibly because a fraction of thiolase forms a tight association with the peroxisome matrix which may contain a cohesive core of proteins (Hayashi *et al.*, 1981; Alexson *et al.*, 1985; Poole and Crane, 1992). The amount of gGPrA solubilized by Triton X-100 alone was the same as that solubilized by Triton X-100/SDS suggesting that the majority of the passenger protein is soluble in the peroxisome and does not form inclusion bodies. All other treatments solubilized thiolase and the majority of the gGPrA fusions.

Western blot analysis of treated peroxisomes from DL1[gGPrASCTN] show isoforms of the gGPrA-fusion with slightly different molecular weights (Fig. 15) which show different solubilization properties, particularly when trested with high pH (treatments D and E). The origin of these apparent isoforms of gGPrASCTN is unknown and they were not observed in untreated peroxisomes. Trichloroacetic acid precipitation of the fractions to remove detergents and buffer components from the samples did not alter the migration of these polypeptides on SDS-PAGE (not shown).

The conclusions that can be drawn from this experiment are that gGPrA fusions probably do not form inclusion bodies and are not stuck in or on the peroxisomal membrane, nor do they associate strongly with other peroxisomal matrix elements.

Fig. 15. Disassembly of peroxisomes from strains expressing gGPrASKL and gGPrASCTN. Peroxisomes from strains DL1[gGPrASKL] and DL1[gGPrASCTN] were prepared by differential centrifugation and density gradient centrifugation. Aliquots were incubated for 30 min on ice, without additions (A), or with 1% Triton X-100 (B), 1% Triton X-100 and 1% SDS (C), 10 mM Tris-HCl pH 8.5/2.5 mM EDTA/500 mM NaCl (D), and 100 mM sodium carbonate pH 11.0 (E). The incubated samples were fractionated into a 100,000 X *g* supernatant (S) and pellet (P). The proteins were resolved by SDS-PAGE on 10% gels. nitrocellulose blots were divided into upper and lower segments. The upper blots were probed with anti-*S. cerevisiae* thiolase serum followed by AP-conjugated anti-rabbit IgG. Lower segments were probed with non-specific AP-conjugated rabbit IgG. Immunoreactive bands were detected with NBT/BCIP. Open arrow = thiolase. Filled arrow = gGPrA constructs.

One of the possible reasons proposed for the inability of the PrA domain alone to be competent for translocation in several *in vitro* experiments was that the addition of the 27-amino acid segment, gG, influenced the folding state of the PrA domain (Janiak *et al.*, 1994). In order to test whether the gG segment influenced peroxisomal targeting of PTS1 and PTS2 fusions, each of the plasmids used in the *in vivo* targeting experiments described above were digested with *Nco*I and recircularized with ligase. This action created the vectors pPrASTOP, pPrASKL, pPrAAKI, and pPrASCTN from which the DNA encoding gG was deleted.

When analyzed by subcellular fractionation no difference was observed between the results obtained for PrA and gGPrA (Fig. 16). Therefore, if the gG segment influences the conformation of the PrA domain, the difference in conformation does not appear to influence targeting to peroxisomes in yeast.

Experimentally it has been demonstrated that the information for correct routing of *S. cerevisiae* thiolase to peroxisomes is contained within the N-terminal 16 amino acids which includes residues that are highly conserved among rat thiolase and a number of other peroxisomal proteins thought to have PTS2 motifs (see Table 2). In addition, gGPrA was shown to be a passive passenger protein, superior to both CAT and DHFR in functionally displaying both C- and N-terminal targeting signals and following the indicated translocation pathway with fidelity. The inclusion of the 27-amino acid gG segment required for translocation competence in other targeting assays had no influence on the translocation on the PrA domain to peroxisomes.

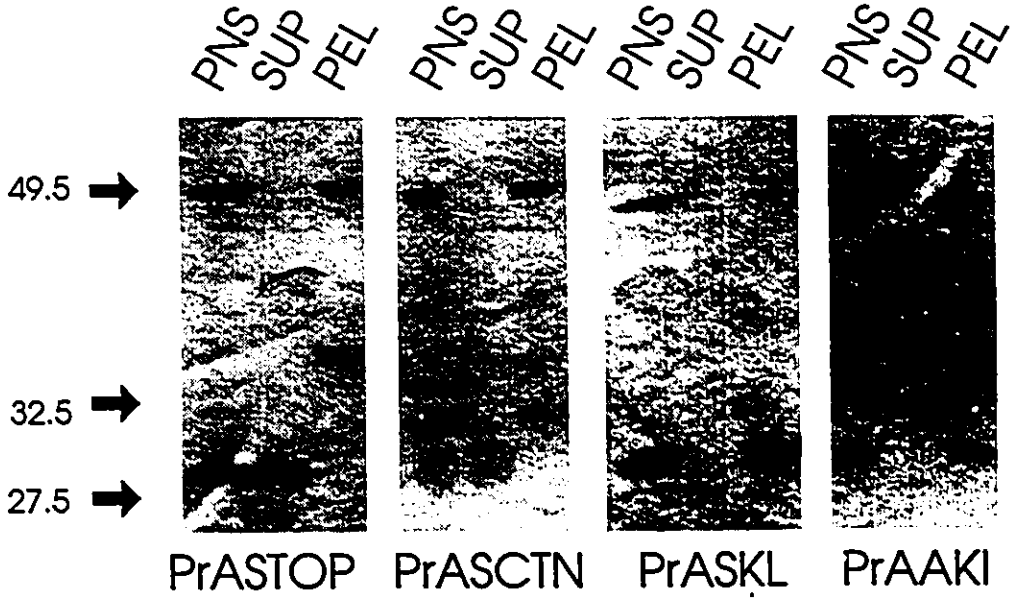

Figure 16. Western blot analysis of subcellular fractions of yeast strains expressing PrA unfused, and fused to PTS1 and PTS2 signals. 500 mL cultures of DL1[PrASTOP], DL1[PrASKL], DL1[PrAAKI], and DL1[PrASCTN] were grown for 18 h in SCIM. Equivalent proportions of post nuclear supernatant (PNS), 20,000 x *g* supernatant (SUP), and 20,000 x *g* pellet (PEL) were resolved by SDS-PAGE on a 10% gel. The proteins were transferred onto nitrocellulose. The blots were probed with anti-*S. cerevisiae* thiolase serum followed by AP-conjugated anti-rabbit IgG. The blot was also probed with non-specifc AP-conjugated rabbit IgG. The immunoreactive bands were detected with NBT/BCIP. M_r of standards is given in kDa.

3.2. UNLIKE PEROXISOMAL THIOLASES IN OTHER SPECIES, THE SIGNAL SEQUENCE OF *S. CEREVISIAE* THIOLASE IS NOT CLEAVED

One of the distinguishing characteristics of peroxisomal thiolase compared to the majority of other peroxisomal matrix proteins is that the protein is cleaved upon import (Fujiki *et al.*, 1984; Preisig-Müller and Kindl, 1993; Nuttley *et al.*,1994) and that the targeting information is contained on the cleaved N-terminal segment (Swinkels *et al.*, 1991). Glyoxysomal malate dehydrogenase which has an N-terminus resembling that of thiolase is one of the few microbody proteins other than thiolase which is also cleaved on import (Yamaguchi *et al.*, 1987).

The primary role of cleavable presequences is to mediate the engagement of the targeted protein with components of the translocation machinery which initiate import. For proteins co-translationally translocated to the endoplasmic reticulum this process is initiated by the interaction of the ribonucleoprotein signal recognition particle (SRP) with the signal sequence as the nascent polypeptide emerges from the ribosome. For mitochondrial targeting signals the presequence may interact with cytolsolic factors such as the presequence binding factor (PBF; Murakami *et al.*, 1988; Murakami and and Mori, 1990).

An additional role for the cleavable presequence is that it may inhibit folding of the preprotein prior to translocation. *In vitro* experiments in which the folding kinetics of preproteins *versus* their mature counterparts provide evidence, for example, that β-lactamase precursor folds 15 times more slowly than the mature protein (Laminet and

Plückthorn, 1989). Similarly the presence of leader sequence slows the folding of ribose- and maltose-binding proteins by a factor of 3 (Park *et al.*, 1988). Interactions between the leader sequence and the bulk of the protein that modulates folding can favour the retention of an import-competent conformation of post translationally translocated proteins until translocation has occurred. The cleavage of the translocated protein then favours the folding of the mature protein into its final active conformation.

In order to determine if the peroxisomal thiolase of *S. cerevisiae* was processed during import to peroxisomes, the full-length coding region was transcribed and translated in a cell-free rabbit reticulocyte lysate and the translation product compared to authentic thiolase from purified peroxisomes (Fig. 17). No difference in the size of the thiolases could be seen in this test although the cleavage of a small number of amino acids would probably not be detected.

The possibility remained that the strain used in these studies (DL1) was defficient in the activity responsible for thiolase cleavage in other organisms. Therefore the thiolase in a panel of *S. cerevisiae* strains with various genetic backgrounds was analyzed by SDS-PAGE and western blotting. Again, with respect to molecular size, thiolases from each strain were essentially identical to that of DL1 (Fig. 18).

An important observation in this experiment is that the thiolase of the yeast strain YPH1 was found entirely in the cytosolic fraction. The same fractions probed with anti-SKL antiserum indicated that several proteins which react with this serum and which are peroxisomal (Aitchison *et al.*, 1992) were also mislocalized to the cytosolic

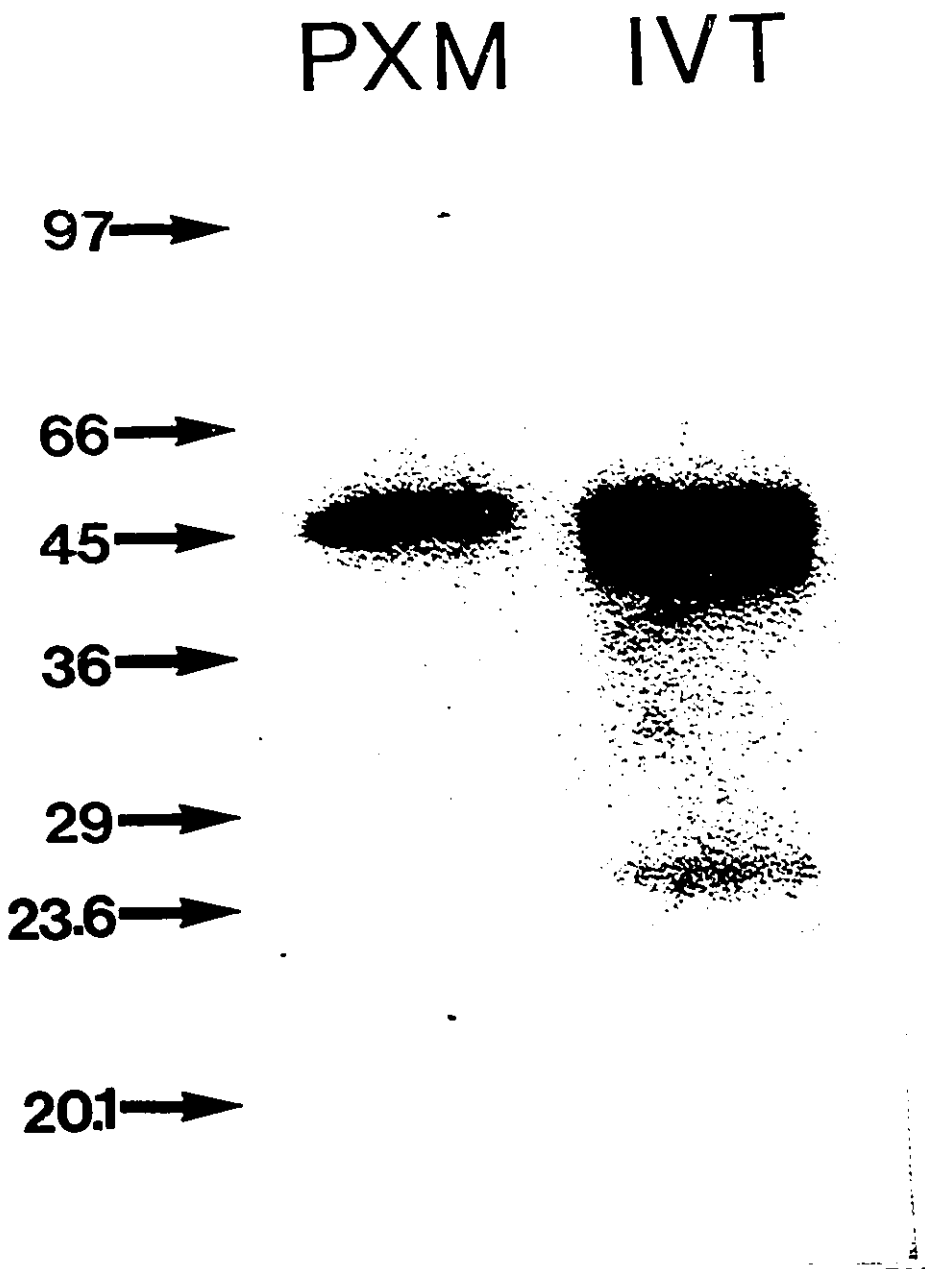

Figure 17. SDS-PAGE analysis of *in vivo* and *in vitro* synthesized thiolase. mRNA for *S. cerevisiae* thiolase was prepared by *in vitro* transcription with SP6 RNA polymerase and translated *in vitro* in rabbit reticulocyte lysate in the presence of ^{35}S-methionine. 5 µg of purified peroxisomes from DL1 (PXM) and a 5 µL aliquot of the translation reaction (IVT) were resolved by SDS-PAGE on a 10% gel. The proteins were transferred onto Nytran™ and the blot was probed with anti-*S. cerevisiae* thiolase serum followed by ^{125}I-Protein A. The blot was dried and soaked in 10% PPO dissolved in toluene, dried, and exposed to X-ray film at -70°C. The M_r of ^{14}C-molecular weight markers is given in kDa.

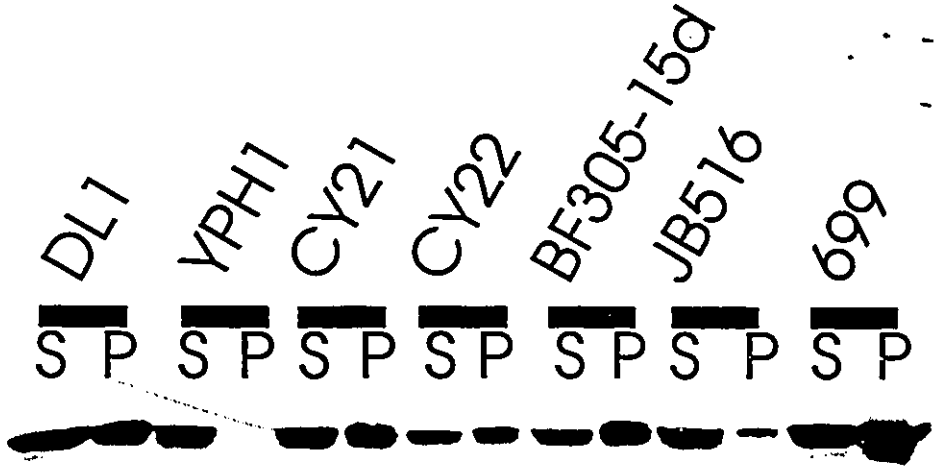

Figure 18. SDS-PAGE analysis of thiolase from diverse strains of laboratory yeasts. 10 mL cultures of the indicated strains were grown in SCIM. An equivalent portion of each 20,000 x *g* supernatant (S) and 20,000 x *g* pellet (P) was resolved by SDS-PAGE on a 10% gel. The proteins were transferred to nitrocellulose and the blots were probed with anti-*S. cerevisiae* thiolase serum followed by AP-conjugated anti-rabbit IgG. Immunoreactive bands were detected with NBT/BCIP.

fraction suggesting that this yeast has a global peroxisomal assembly defect (Fig. 19). A previous observation of a peroxisome assembly defect in the laboratory strain "YP102" has been reported (Van der Leij *et al.*, 1992). Recently the sequence of the PAS8 gene responsible for the peroxisome assembly defect in this yeast was published (Voorn-Brouwer *et al.*, 1993). The strain was reported to have the same genotype as YPH102, a strain derived from YPH1 by Sikorski and Hieter (1989) and was probably consistently misnamed in these reports. It is likely that all the strains derived from YPH1 exhibit the *pas*8 phenotype.

The observation that thiolase in a peroxisome assembly mutant of *S. cerevisiae* has the same subunit molecular size as in the strain with normal peroxisome assembly characteristics stands in contrast to the *Y. lipolytica pay*4 strain in which the cytosolic thiolase is clearly of a higher molecular weight than the peroxisomal thiolase in the wild type strain (Nuttley *et al.*, 1994). Two possibilities can account for this. Either *S. cerevisiae* has lost the processing capability, or the sequence of *S. cerevisiae* thiolase corresponding to the cleavage site has diverged from its ancestral prototype such that it is no longer recognized as a substrate for the cleavage event.

Nothing is currently known about the cleavage activity which processes preproteins in the peroxisomal matrix. The glyoxysomal malate dehydrogenase (gMDH) from watermelon is processed in plant glyoxysomes (Yamaguchi *et al.*, 1987; Gietl, 1990) and is similar to peroxisomal thiolases with respect to the PTS2 consensus sequence (see Table 2). When expressed in *H. polymorpha*, gMDH is targeted to

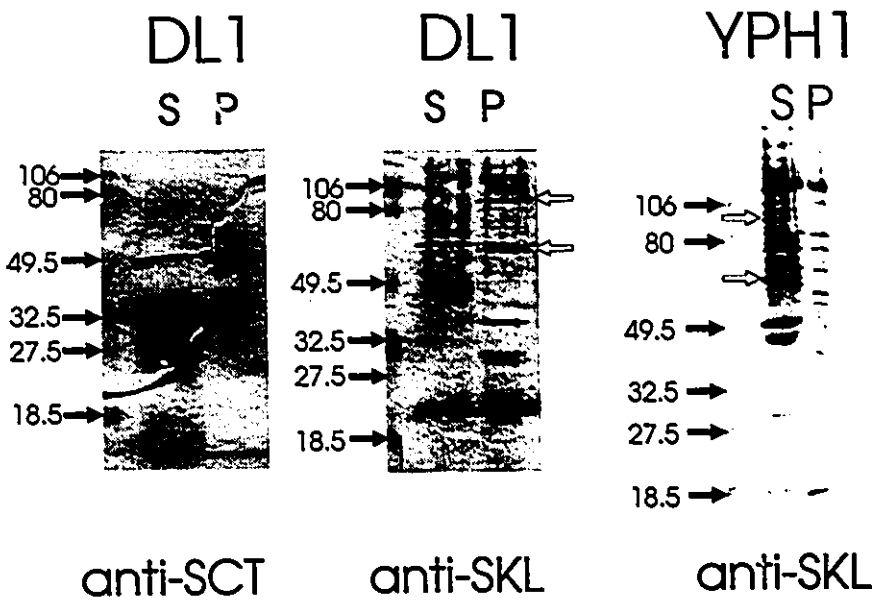

Figure 19. Western blot analysis of subcellular fractions from normal (DL1) and peroxisome-deficient (YPH1) laboratory strains. 10 mL cultures of DL1 and YPH1 were grown in SCIM for 20 h. Equal portions of the 20,000 x *g* supernatant (S) and 20,000 x *g* pellet (P) were resolved by SDS-PAGE on 10% gels and proteins transferred to nitrocellulose. Blots were probed with either anti-*S. cerevisiae* thiolase or anti-SKL sera followed by AP-conjugated anti-rabbit IgG. Immunoreactive bands were visualized with NBT/BCIP. Major SKL-reactive bands (96 and 54 kDa) are indicated by open arrows. M_r of BioRad molecular weight standards is given in kDa.

peroxisomes, is enzymatically active, but is not processed into its mature form (van der Klei *et al.*, 1993).

To summarize, cleavage of thiolase was not detected in *S. cerevisiae*. The absence of cleavage in *S. cerevisiae* raises interesting questions as to the distribution of this trait in other yeasts and other eukaryotic lineages.

3.3. THIOLASE-DEFICIENT STRAIN OF *S. CEREVISIAE*: STUD

In order to accurately ascertain the targeting function of thiolase with amino acid substitutions within the PTS2, a thiolase deficient strain of *S. cerevisiae* was constructed using homologous recombination. The benefit of using such a strain is two-fold. First, The deletion of the chromosomal gene encoding *S. cerevisiae* thiolase would allow the analysis of subcellular distribution of plasmid-encoded thiolases by western blot analysis without interference from the chromosomally encoded polypeptide. Secondly, because the β-oxidation of fatty acids in *S. cerevisiae* is restricted to the peroxisome (Kawamoto *et al.*, 1978; Kunau *et al.*, 1988), deletion of the peroxisomal thiolase should result in a recessive negative phenotype– the inability to grow on oleic acid as the sole carbon source. Plasmid encoded thiolases that are correctly targeted to peroxisomes should have a dominant effect and restore growth on oleic acid, whereas those which are mistargeted should not. The two methods of analysis should yield complementary results.

Randomly selected uracil prototrophs were further tested for their ability to grow

on oleic acid and to express chromosomally encoded thiolase (not shown). Two strains STUD3.4 and STUD5.8 differing only in the orientation of the *URA3* gene were selected (STUD = *Saccharomyces cerevisiae* thiolase *URA3* disruption).

Southern blot analysis of genomic DNA from strain STUD3.4 (this strain was used in subsequent experiments and was designated STUD for simplicity) indicated the presence of *Sau*3AI fragments which hybridize with both *S. cerevisiae* thiolase coding region and *URA3* probes corresponding to the 943 and 501 bp predicted for the *URA3* gene inserted into the chromosomal *POT1* gene encoding thiolase (Fig. 20, lanes 4 of both blots).

Strains STUD3·4 and STUD5·8 were transformed with pSG524, pSG522, and pSCTΔN and the expression of thiolase was analysed by western blot in total cell lysates (Fig. 21). As expected thiolase was detected only in the parental strain DL1 and in plasmid-containing transformants.

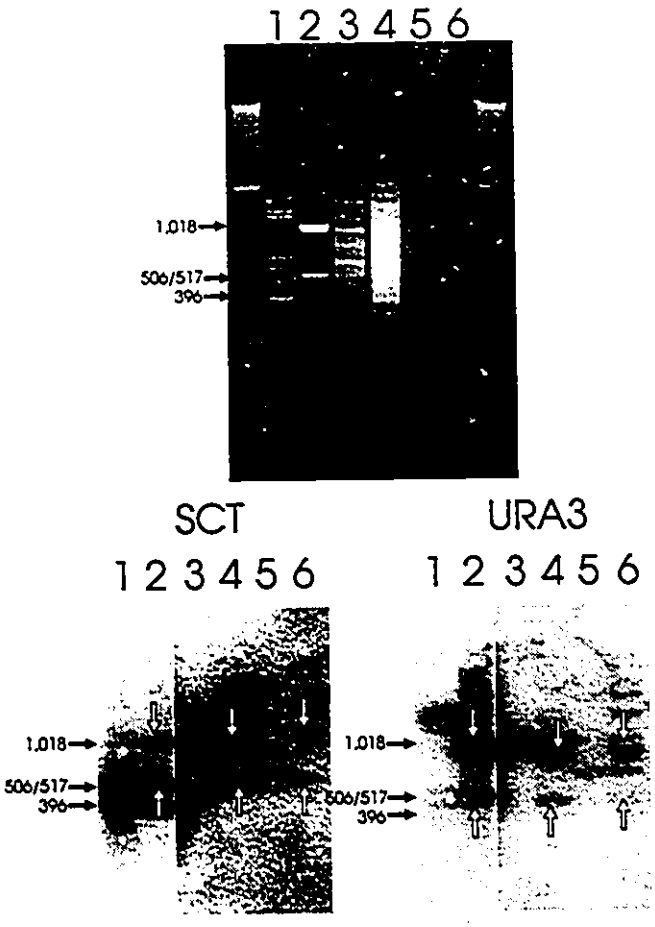

Figure 20. Southern blot analysis of genomic DNA isolated from parent strains and thiolase knockout strains. Genomic DNA was prepared from 10 mL cultures of DL1 (lane 3), STUD (lane 4), YPH102 (lane 5), and YPHSTUD (lane 6) grown in YEPD. The plasmids pSG524 (lane 1) and pSTUD3.3 (lane 2), along with 20 μg of genomic DNA were digested with *Sau*3AI and resolved by electrophoresis on a 1% agarose gel (ethidium bromide-stained gel shown). Blots were probed with labelled DNA specific for thiolase (SCT) or the *URA3* sequence. Molecular sizes of 1 kb ladder markers are given in bp. Arrows highlight diagnostic fragments which hybridize with both probes.

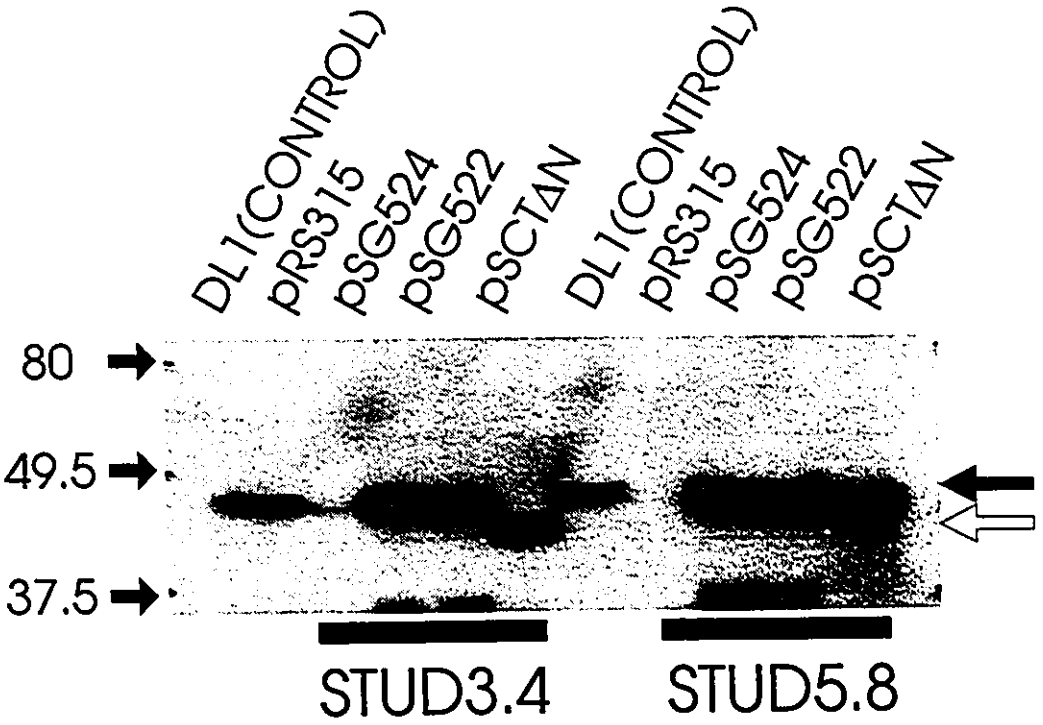

Figure 21. Western blot analysis of thiolase expression in STUD. 10 mL cultures of STUD, STUD[524], STUD[522], and STUD[ΔN] were grown for 18 h in SCIM. Glass bead lysates were resolved by SDS-PAGE on a 10% gel. The proteins were transferred onto nitrocellulose and the blots were probed with anti-*S. cerevisiae* thiolase serum followed by HRP-conjugated anti-rabbit IgG antibody. The immunoreactive bands were visualized by ECL™ with a 10 s exposure on X-ray film. Filled arrow = full-length thiolase. Open arrow = truncated thiolase. M_r of standards is given in kDa.

3.4. SUBSTITUTION OF CONSERVED RESIDUES WITHIN THE PTS2 OF THIOLASE INFLUENCES THE EFFICIENCY OF IMPORT INTO PEROXISOMES.

A comparison of peroxisomal thiolases from several sources reveals a conservation amongst all thiolases of certain amino acids found within the 11-amino acid peroxisomal targeting signal of rat thiolase (see Table 2). In the *S. cerevisiae* PTS2 region, residues which are identical in mammalian and yeast peroxisomal thiolases are Arg4, Leu5, and Leu12. His11 and Gln6, which are identical among *S. cerevisiae* thiolase and the mammalian thiolases and conservatively substituted in the thiolases from other organisms, were also targeted for mutagenesis. The importance of these amino acids in targeting *S. cerevisiae* thiolase to peroxisomes was determined by saturation mutagenesis of the corresponding codons, followed by expression of the mutant thiolase genes *in vivo*.

Subcellular localization of mutant thiolases was determined using two assays. The first was a functional assay involving growth of *S. cerevisiae* on oleic acid-medium. The thiolase gene knockout strain STUD and the STUD strain carrying a plasmid expressing the gene coding for Δ1-16 thiolase cannot grow on oleic acid-medium, while the STUD strain carrying a plasmid expressing the wild-type thiolase gene can grow on this medium (Fig. 22). Therefore, growth on oleic acid-medium necessitates correct targeting of thiolase to peroxisomes. The second assay is biochemical, involving subcellular fractionation followed by immunodetection with anti-thiolase antibodies (Fig. 23).

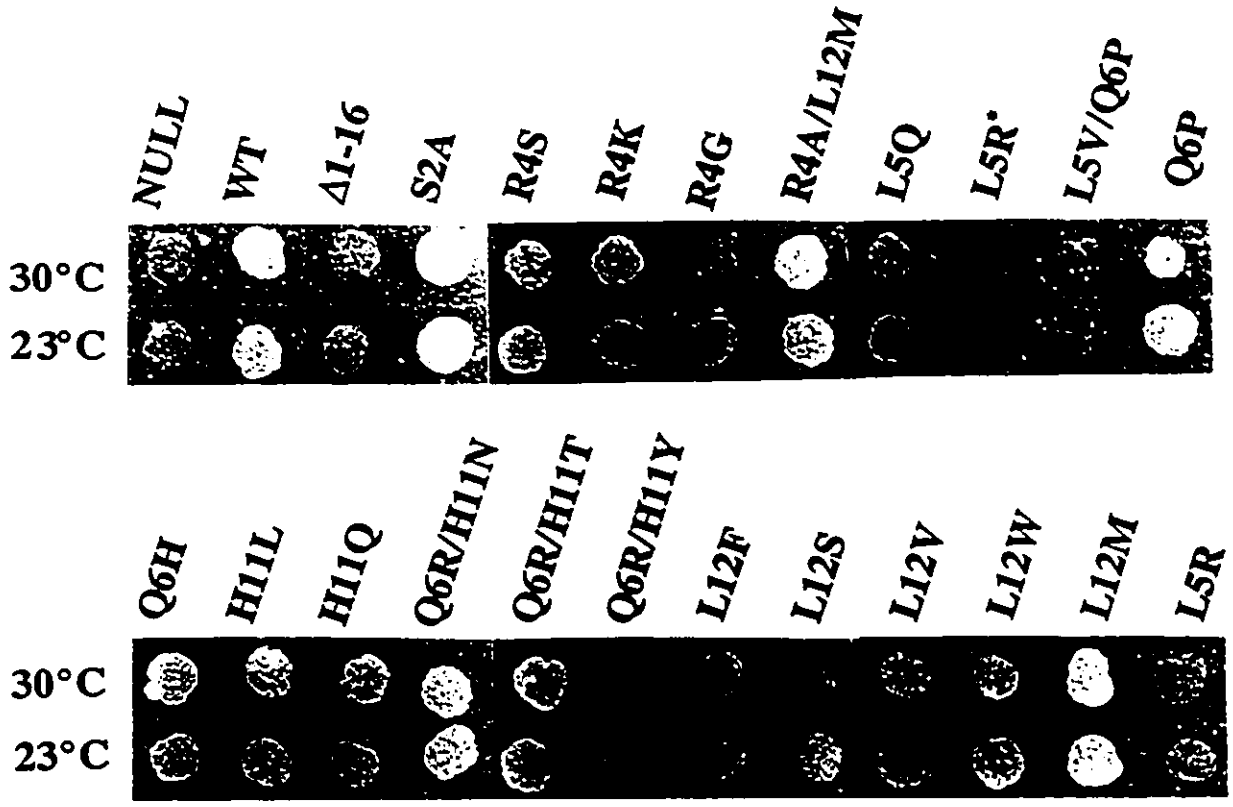

Figure 22. Growth of STUD complemented by wild-type thiolase and thiolases with mutations in the PTS2 region. Cultures of the indicated strains were grown to saturation in YNBD. Each culture was diluted 10-fold in sterile water, and a 2 μL aliquot was applied to the surface of YNO plates. The plates were incubated at the indicated temperatures for 10 days and photographed.

Figure 23. Western blot analysis of subcellular fractions of STUD expressing wild-type thiolase and thiolases with mutations in the PTS2 region. 10 mL cultures of the indicated strains were grown at 30°C for 18 h in SCIM. An equal portion of each 20,000 x *g* supernatant (S) and 20,000 x *g* pellet (P) was resolved by SDS-PAGE on 10% gels. Proteins were transferred to nitrocellulose and the blots were probed with anti-*S. cerevisiae* thiolase serum followed by ^{125}I-Protein A. The blot was exposed on a phospor storage screen.

A detailed listing of the sequences of mutants that were transformed into STUD is given in Table 6.

Table 6. Sequences of PTS2 mutants of thiolase.

WT	TCT	CAA	AGA	CTA	CAA	AGT	ATC	AAG	GAT	CAT	TTG	GTG	GAG	AGC	GCC	WT
pSG522	G--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	S2A
Clone 1	---	---	--C	--T	---	---	---	---	---	---	---	---	---	---	---	R4S
Clone 10	---	---	---	---	-C-	---	---	---	---	---	---	---	---	---	---	Q6P
Clone 15	---	-**	***	***	***	***	*--	---	---	GG-	---	---	---	---	---	Δ3-8L/H11G
Clone 37	---	---	---	---	---	---	---	---	---	--G	---	---	---	---	---	H11Q
Clone 44	---	---	---	--G	---	---	---	---	---	-T-	---	---	---	---	---	H11L
Clone 54	---	---	---	CG-	---	---	---	---	---	---	C--	---	---	---	---	L5R*
Clone 59	---	---	---	CG-	---	---	---	---	---	---	---	---	---	---	---	L5R
Clone 60	---	---	---	---	---	---	---	---	---	---	G--	---	---	---	---	L12V
Clone 64	---	---	---	---	--T	---	---	---	---	---	---	---	---	---	---	Q6H
Clone 69	---	---	---	---	---	---	---	---	---	-**	***	***	*--	---	---	Δ11-14Q
Clone 72	---	---	---	---	---	---	---	---	---	---	-C-	---	---	---	---	L12S
Clone 84	---	---	---	--T	---	---	---	---	---	---	--C	---	---	---	---	L12F
Clone 90	---	---	C--	---	---	---	---	---	---	---	A--	---	---	---	---	L12M
Clone 102	---	---	---	---	-G-	---	---	---	---	T--	---	---	---	---	---	Q6R/H11Y
Clone 108	---	---	---	---	-G-	---	---	---	---	A--	---	---	---	---	---	Q5R/H11N
Clone 116	---	---	---	-A-	---	---	---	---	---	---	---	---	---	---	---	L5Q
Clone 128	---	---	---	---	-GG	---	---	---	---	A--	---	---	---	---	---	Q6R/H11T
Clone 135	---	---	-A-	---	---	---	---	---	---	---	---	---	--A	---	---	R4K
Clone 136	---	---	---	---	---	---	---	---	---	-T-	---	---	---	---	---	H11L
Clone 159	---	---	G--	---	---	---	---	---	---	---	---	---	---	---	---	R4G
Clone 171	---	---	---	---	---	---	---	---	---	---	-G-	---	---	---	---	L12W
Clone 172	---	---	---	---	---	---	---	---	---	---	A--	---	---	---	---	L12M
Clone 173	---	---	---	--G	---	--*	***	***	***	**-	---	---	---	---	---	Δ8-11N

Random clones were selected and sequenced. The wild type sequence of codons for amino acids 2 to 15 is given in the top line. Dashes indicate unchanged nucleotides. Asterisks indicate deleted nucleotides.

Each of 15 nucleotide positions in 5 codons had a 0.1 probability of incorporating an altered nucleotide. Ideally the majority of mutations resulting from this method would contain single nucleotide substitutions along with some double substitutions. In practice, the overall results of the mutagenesis strategy were as follows: wild type including silent mutations, 13%; mutations in single codons resulting in amino acid substitutions, 19%; mutations in two codons resulting in substitutions, 9%; mutations resulting in premature stops, 5%; miscellaneous defects including no initiation codon, deletion of one or more

bases, and one or more base changes in codons not targeted for mutagenesis, 54%. The high percentage of miscellaneous defects may be attributable to difficulties in purifying the synthetic oligonucleotide reported by the manufacturer due to its length and heterogeneity of sequence.

Three clones (15, 69, and 173), all containing deletions within the PTS2 domain, were examined in preliminary screens and found to be mislocalized to the cytosolic fraction and were not examined further (not shown).

The inability of the STUD strain expressing certain mutant thiolase genes to grow on oleic acid-medium was not due to the synthesis of enzymatically inactive thiolase in these transformants. All mutant thiolases were enzymatically active (Table 7) and at levels comparable to that of wild-type thiolase. The specific activities of mutant thiolases varied between 2.8 and 11.0 nmol·min^{-1}·mg protein^{-1}. The specific activity of wild-type thiolase was 6.6 nmol·min^{-1}·mg protein^{-1}. No thiolase activity was detected either immunologically or enzymatically in the STUD strain transformed with the parental vector pRS315 (designated "NULL").

One mutant, L5R*, did not grow on oleic acid-medium and showed almost no thiolase activity (0.14 nmol·min^{-1}·mg protein^{-1}); however, this low activity was due not to the production of normal levels of a poorly active thiolase but to the low levels of thiolase synthesized (Fig. 23). Re-examination of the sequence of this clone disclosed an unexpected deletion of two Cs at positions -2 and -1 in the 5' untranslated region (where the A of the ATG initiation codon is designated +1). The occurrence of specific

Table 7. Wild-type and mutant thiolase strains: subcellular fractionation, thiolase activity, and growth on oleic acid-medium

STRAIN	20kgS 20kgP (% of total)	GROWTH @23°C @30°C	ACTIVITY (nmol·mg^{-1}·min^{-1})
NULL	n.d.	- -	n.d.
WT	15 85	+ +	6.6
Δ1-16	97 3	- -	4.7
S2A	14 86	+ +	4.9
R4S	90 10	+ +	6.5
R4K	65 35	+ +	10.3
R4G	95 5	+ -	8.0
R4A/L12M	14 86	+ +	7.7
L5Q	90 10	+ +	10.9
L5R*	88 12	- -	0.14
L5V/Q6P	92 8	- -	10.7
Q6P	70 30	+ +	8.2
Q6H	24 76	+ +	11.0
H11L	27 73	+ +	4.7
H11Q	49 51	+ +	5.6
Q6R/H11N	27 73	+ +	5.6
Q6R/H11T	34 66	+ +	6.3
Q6R/H11Y	60 40	+ -	5.5
L12F	28 72	+ +	8.1
L12S	91 9	+ -	2.8
L12V	12 88	+ +	6.9
L12W	55 45	+ +	6.1
L12M	11 89	+ +	8.8
L5R	91 9	- -	6.3

The percent distribution of thiolase in the 20 kgP (20,000x *g* pellet) and 20 kgS (20,000 x *g* supernatant) was determined by western blot analysis with ^{125}I-protein A, followed by quantitation on a phosphorimager. Growth on YNO agar was assessed after incubation at the indicated temperature for 10 d. Enzyme activity was determined as described in Materials and Methods (cultured at 30°C). n.d. = not detected

nucleotides in the region immediately adjacent to an initiation codon can influence the expression of the encoded protein (Cigan and Donahue, 1987). It is therefore most likely that the small deletion reduces translation initiation in L5R*. A second mutant, L5R, still could not grow on oleic acid-medium (Fig. 22), even though it made increased amounts of thiolase (Fig. 23) with normal enzymatic activity (6.3 nmol·min^{-1}·mg protein^{-1}).

Only the mutant L5R and the double mutant L5V/Q6P resulted in the inability to grow on oleic acid-medium at either 23°C or 30°C (Fig. 22) and in the almost total mislocalization of thiolase to the cytosol (Fig. 23). Q6P alone results in a moderate reduction in targeting efficiency, and the Leu to Val substitution at position 5 is conservative and might therefore be expected to result in little or no further diminution of targeting over that observed in Q6P. However, it appears that these side-by-side substitutions act synergistically to abolish targeting and import of thiolase into peroxisomes.

The small amounts of thiolase found in the organellar pellets of nongrowing yeast may be attributed to cytosolic thiolase that is trapped nonspecifically in the organellar pellet during subcellular fractionation. The organellar fractions of four mutants (R4S, R4G, L5Q, L12S) have thiolase in amounts similar to those of mutants that do not support growth. Therefore, it is probable that only a small amount of thiolase needs to be correctly targeted to peroxisomes to support growth on oleic acid medium.

Two mutants (R4G and L12S) targeted thiolase poorly. These mutants were unable to grow at 30°C but were able to grow at 23°C on oleic acid-medium. This

temperature sensitivity may result from thermal destabilization within the thiolase peroxisomal targeting signal, resulting in reduced interaction between it and factor(s) involved in the recognition of the signal.

Three single (S2A, L12V, L12M) and one double (R4A/L12M) mutation had negligible effects on targeting and did not detectably effect growth on oleic acid medium. The conversion of Ser 2 to Ala (S2A), through the introduction of an *Nco*I site at the initiation codon of the thiolase gene in pSG522, was not expected to affect the targeting of thiolase, as this amino acid is not conserved in the different thiolases. The mutations L12V and L12M were tolerated, most likely because the substituted amino acids occupy the same approximate volume as Leu and because they maintain hydrophobicity at this position. In contrast, mutants with bulky aromatic side chains at L12 (L12F and L12W) were less efficiently targeted and grew more poorly on oleic acid medium than either L12V and L12M. The larger aromatic side chains may cause steric interference between the thiolase targeting signal and the presumed PTS2 import receptor.

The double-mutant R4A/L12M was efficiently targeted and supported strong growth on oleic acid medium. This result is difficult to explain given that the more conservative substitution R4K was less efficiently targeted and had decreased growth on oleic acid-medium. An Arg to Lys substitution in the PTS2 of rat thiolase had a similar effect on peroxisomal targeting in mammalian cells (Tsukamoto *et al.*, 1994). If the reduced volume of the Ala side chain compensated for the loss of the positively charged Arg side chain, then the mutant R4S should be expected to be accommodated as well as

R4A. However, this is not the case as the mutant R4S was poorly targeted. The possibility remains that the second amino acid mutation (L12M) in the double-mutant compensates in an unknown way for the loss of Arg at position 4.

All known thiolases have a conserved Gln or Asn corresponding to position 6 in *S. cerevisiae* peroxisomal thiolase. Mutation of Gln at this position to Pro (Q6P) resulted in reduced targeting of the mutated thiolase *vis-à-vis* wild-type thiolase. This reduced targeting might be the result of the loss of hydrogen bonding-capacity at this position in the mutant. The mutant Q6H retains hydrogen bonding-capacity and was more efficiently targeted than Q6P but still less well than wild-type thiolase. In contrast, thiolase in which the conserved His at position 11 is replaced by Gln (H11Q) was divided almost evenly between the cytosolic and organellar fractions. The mutant H11L was targeted more efficiently than H11Q.

Of the three double-mutants encompassing Q6 and H11 (Q6R/H11N, Q6R/H11T, and Q6R/H11Y), Q6R/H11Y is most interesting in that while approximately 40% of thiolase was localized to the organellar fraction in yeast grown at 30°C, this mutant was incapable of growth on oleic acid-medium at 30°C. These crude fractionation results do not distinguish between the peroxisomal thiolase and thiolase which may be mislocalized to one or more compartments which cofractionate with peroxisomes in the organellar pellet. Strong immunofluorescence of cytosolic thiolase does not permit identification of particular pattern of localization of the Q6R/H11Y mutant. Density gradient centrifugation indicates a substantial association between peak cytochrome *c* oxidase

activity (a mitochondrial marker enzyme) and Q6R/H11Y thiolase detected by western blotting greater than the level of contamination of the mitochondria normally observed in the fractionation of yeast expressing WT thiolase (Fig. 24). It is possible that mutations in His11 of *S. cerevisiae* thiolase can have similar effects as mutation of His-17 of rat peroxisomal thiolase B to Arg, Lys, Leu, or Val, which result in dual localization of DHFR-PTS fusion to both mitochondria and cytosol in CHO cells (Osumi *et al.*, 1992; Tsukamoto *et al.*, 1994).

In order to establish whether certain mutant PTS2s could act as mitochondrial targeting signals, the yeast strain YPH102 (Sikorski and Hieter, 1989) a strain derived from YPH1 which has a peroxisomal assembly defect (see above), was transformed with fragments of pGSTUD3 as described above. Eighteen uracil prototrophs were analyzed for expression of thiolase by western blotting of total cell lysates (Fig. 25). Four of the transformants expressed no thiolase and one, YPHSTUD·10 was used for Southern blot analysis to confirm the insertion of the *URA3* gene into the *POT1* locus (see Fig. 20, lanes 6 on both blots). YPHSTUD·10 was used for further experiments and is referred to as YPHSTUD.

YPHSTUD was transformed with all the plasmids carrying mutations at His11 and cytosolic and organellar fractions were analysed by western blotting to determine the subcellular distribution of thiolase polypeptides (Fig. 26). In all cases except one (Q6R/H11Y), most of the thiolase detected in the pellet in STUD was cytosolic in YPHSTUD, suggesting that these proteins were targeted exclusively, but inefficiently,

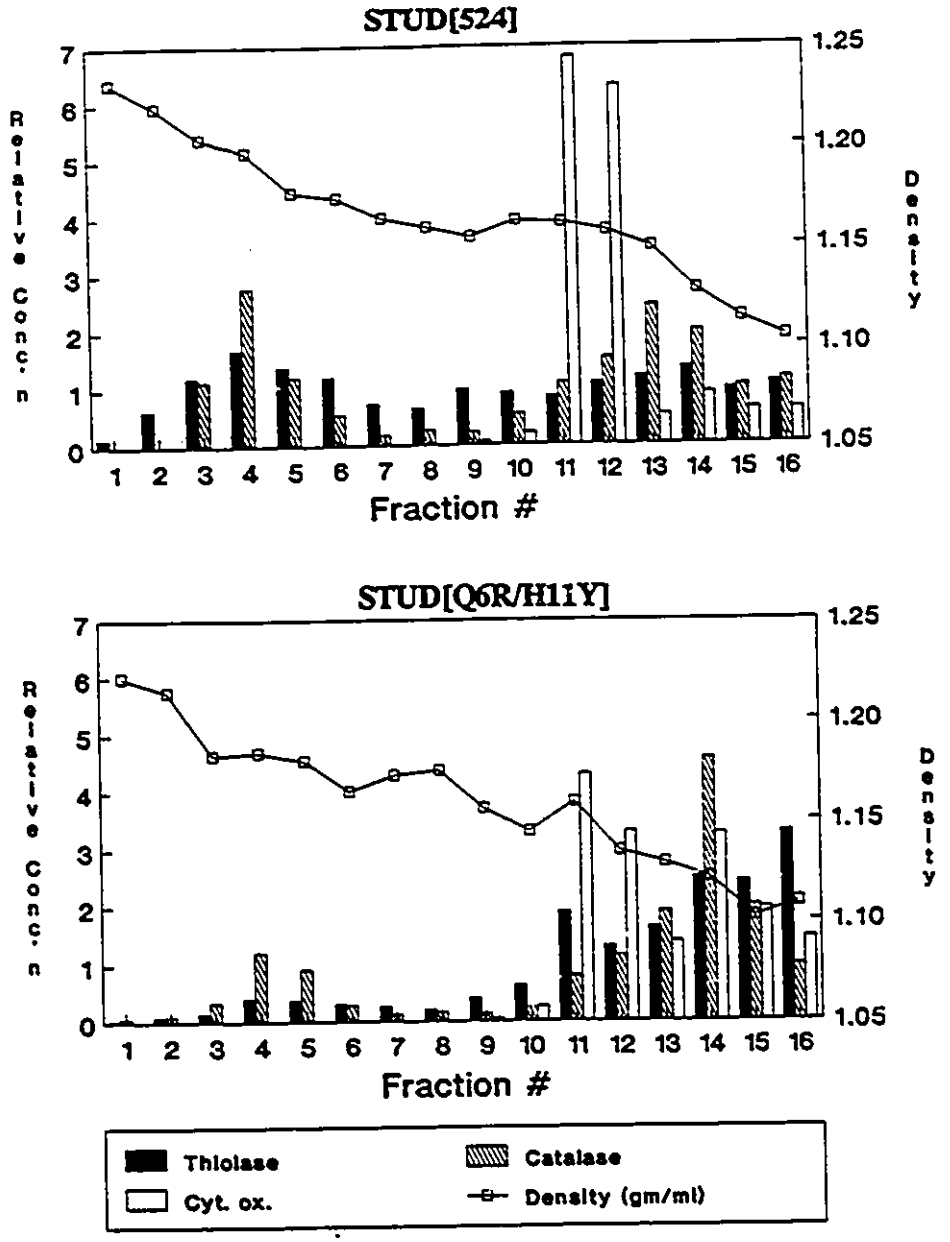

Figure 24. Density gradient fractionation of the organellar fractions of STUD expressing either wild type thiolase or targeting mutant Q6R/H11Y. 500 mL cultures of STUD[524] and STUD[Q6R/H11Y] were grown for 20 h in SCIM. The 20,000 x g pellet was further fractionated on a discontinuous Nycodenz™ gradient. Catalase and cytochrome *c* oxidase activities were measured enzymatically. Thiolase was measured by western blot analysis. The histograms represent the portion in each fraction of the total activity recovered from the gradient.

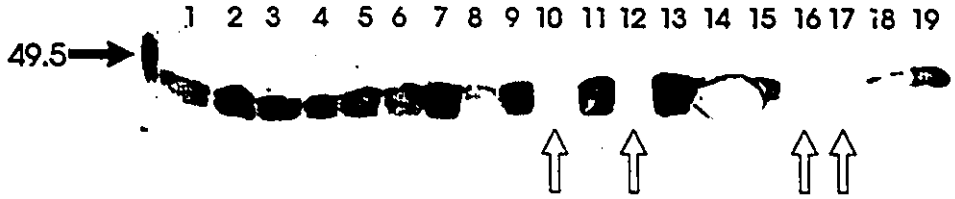

Figure 25. Western blot analysis of uracil prototrophs derived from YPH102 after transformation with thiolase knockout fragments. 10 mL cultures of YPH102 (lane 1) and 18 randomly selected uracil prototrophs were grown for 20 h in SCIM. Glass bead lysates were resolved by SDS-PAGE on a 10% gel and the proteins were transferred to nitrocellulose. The blot was probed with anti-*S. cerevisiae* thiolase serum followed by AP-conjugated anti-rabbit IgG. The immunoreactive bands were visualized with NBT/BCIP. Upward pointing arrows highlight strains which are thiolase deficient.

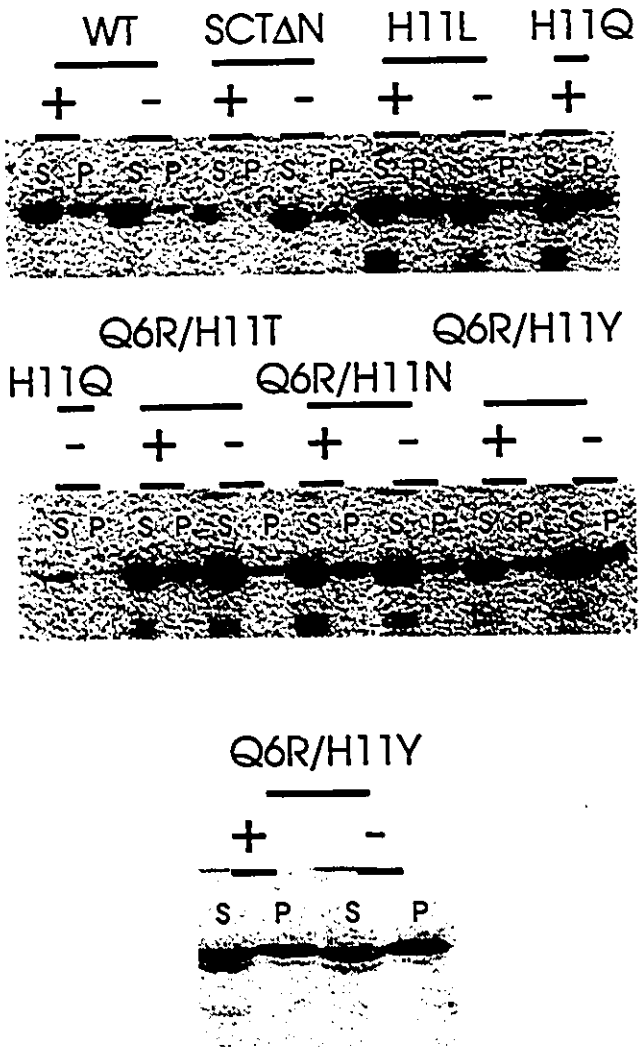

Figure 26. Western blot analysis of subcellular fractions of wild type thiolase and PTS2 mutants expressed in STUD and the thiolase-deficient peroxisome assembly mutant strain, YPHSTUD. Wild type and indicated PTS2 mutants were expressed in both STUD (+ peroxisomes) and YPHSTUD (- peroxisomes). 10 mL cultures were grown for 18 h in SCIM at 30°C. Equivalent proportions of 20,000 x *g* supernatant (S) and 20,000 x *g* pellet (P) were resolved by SDS-PAGE on 10% gels. Proteins were transferred to nitrocellulose and the blots were probed with anti-*S. cerevisiae* thiolase serum followed by ^{125}I-Protein A. The immunoreactive bands were visualized by exposure of the blot to a phosphor storage screen. The experiment was repeated for the strains expressing Q6R/H11Y with the result shown in the bottom panel.

to peroxisomes. The mutant Q6R/H11Y was equally pelletable in YPHSTUD, indicating that the targeting of this protein to a component of the organellar fraction is not dependent on the assembly of normal peroxisomes and strengthens the evidence that it is misdirected inefficiently to mitochondria.

Mutations of amino acids within the minimal targeting sequence defined for rat peroxisomal thiolase have a variety of effects on the localization and import of yeast thiolase as indicated by subcellular fractionation and the ability to restore growth on oleic acid medium in a thiolase-deficient strain. The yield of informative mutants using this particular mutagenesis scheme was less than ideal, and a comprehensive set of mutations in each residue targeted for mutagenesis was not obtained. However, a few important conclusions can be drawn. Substitution of Leu5 by Arg completely abolishes import. A PTS2 mutant with Gln at the same position is severely mislocalized to the cytosol but imported in sufficient quantity to support growth on oleic acid medium. Leu12 is replaced by hydrophobic residues with similar volumes (Met and Val) without significant effect on targeting whereas larger hydrophobic residues (Phe and Trp) reduce targeting efficiency. A polar residue at this location (Ser) abolishes targeting at 30°C. When the N-terminal amino acid sequence of *S. cerevisiae* thiolase is represented on a helical wheel diagram (Fig. 27), Leu 5 and Leu 12, are aligned and may form part of a hydrophobic surface required for interaction of the targeting signal with its receptor. Although our results suggest that hydrophobicity is required at both of these residues, we cannot assume that the targeting sequence forms a helical structure.

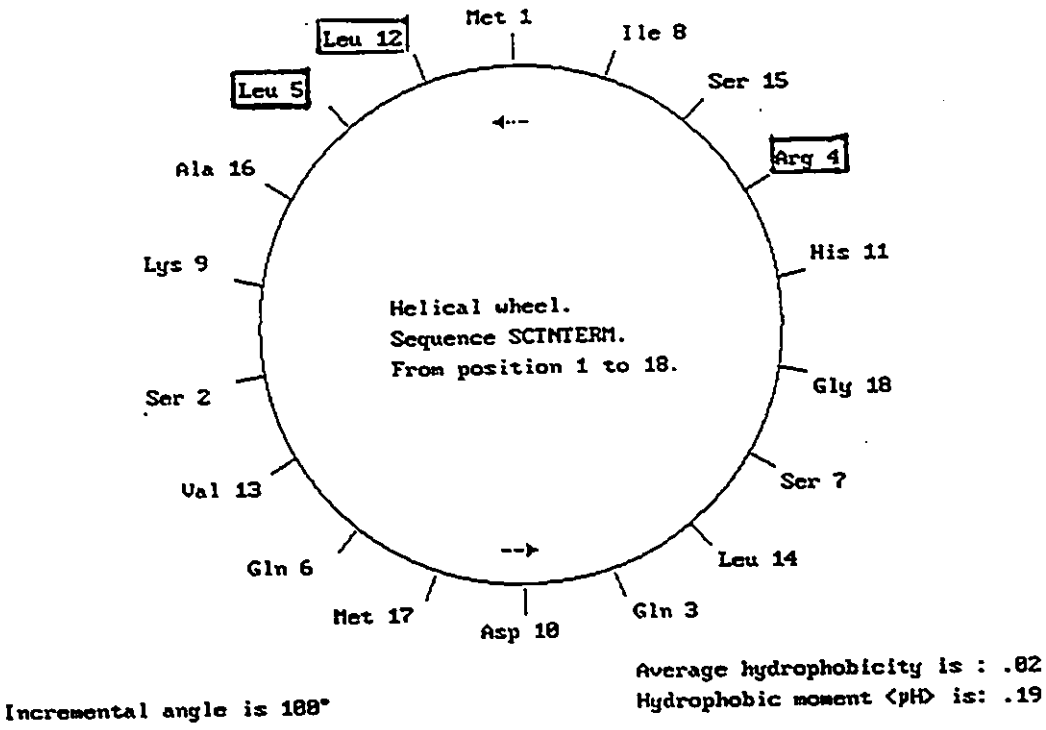

Figure 27. Helical wheel representation of the N-terminus of *S. cerevisiae* thiolase. The N-terminal 18 amino acid residues are schematically presented as an α-helix viewed down the central axis of the helix. Critical residues are boxed.

The severe targeting defect conferred by substitution of Arg4 with either Ser or Gly identifies another critical element of the thiolase PTS2. A conservative substitution (Lys) at this position results in significant mislocalization suggesting that the precise positioning of positively charged residue within the overall structure of the signal is an absolute requirement for maximum function of the targeting signal. Another group working on the PTS2 of rat peroxisomal thiolase has also discovered that substitution of the corresponding Arg residue with Lys reduced import (Tsukamoto *et al.*, 1994).

Analysis of localization of His 11 mutants expressed in YPHSTUD indicates that at least one substitution (H11Y) results in conversion of the PTS2 into an inefficient mitochondrial targeting signal. Although this substitution occurred in a double mutant (Q6R/H11Y), two other His11 mutants with the identical substitution at Gln6 were not targeted to mitochondria (Q6R/H11N and Q6R/H11T). The relatively minor alterations to PTS2 which converts it into a mitochondrial targeting signal observed in this work and by others (Osumi *et al.*, 1992; Tsukamoto *et al.*, 1994) may be entirely fortuitous rather than representing a significant structural similarity between the two types of signal. Mitochondrial signals are diverse and their structural requirements are only broadly defined as rich in basic or hydroxylated residues without long hydrophobic stretches and exhibiting some degree of amphipathicity if arranged as an α-helix or β-sheet (Verner and Schatz, 1988). This description may be applied fairly well to the PTS2 of *S. cerevisiae* thiolase, yet the wild type signal does not confer any substantial degree of mistargeting to mitochondria even in the absence of peroxisome structures (see Fig. 26;

WT).

Restoration of growth on oleic acid medium of a thiolase-deficient strain of yeast by several PTS2 mutants with targeting defects which may be classified as severe, is a sensitive indication that a small fraction of thiolase is imported. This indicates that as long as the majority of the targeting information is present, interaction with the PTS2 receptor is still possible and import can proceed.

3.5. DIMERIZATION PERMITS THE IMPORT OF CYTOSOLIC VARIANTS OF THIOLASE INTO PEROXISOMES.

Early in the effort to characterize the import signal requirements of *S. cerevisiae* thiolase, the plasmid pSCTΔN was constructed and transformed into DL1 with an intact endogenous *POT1* gene encoding full-length thiolase. This strain, DL1[SCTΔN] was therefore programmed to express the full-length *S. cerevisiae* thiolase (M_r calculated: 44764 Da) and the truncated SCTΔN (M_r calculated: 42940). My expectation was that the truncated protein would remain cytosolic and that the full-length protein would be correctly targeted to peroxisomes.

Contrary to this expectation, about 50% of the truncated protein colocalized with the full-length thiolase in the organellar fraction (Fig. 28, panel A). When the organellar fraction was subfractionated on Nycodenz™ gradients, the full-length and truncated thiolases co-migrated precisely (Fig. 28, panel B). Furthermore, protease-protection experiments indicated that both proteins were probably protected inside a structure and

were rendered sensitive to added protease by detergent treatment (Fig. 29).

Following construction of the thiolase-deficient STUD strain, it was observed that the truncated thiolase expressed in the absence of the full-length thiolase was essentially cytosolic (See Fig. 30, STUD[ΔN]). This suggested that at least at the genetic level the full-length thiolase interacted with the truncated thiolase to confer partial import. The native peroxisomal 3-ketoacyl thiolases from a number of sources, including *S. cerevisiae*, occur as dimers composed of identical subunits (Frevert and Kindl, 1980; Miyazawa *et al.*, 1981; Zeelan *et al.*, 1990) Therefore, the most likely model to explain the partial restoration of import of the truncated thiolase in the presence of the full-length thiolase is that the truncated subunits can dimerize randomly with either other truncated subunits or with full-length subunits after synthesis in the cytosol but only dimers with at least a single PTS are competent for import. The random formation of dimers between full-length and truncated subunits expressed at roughly equal levels should result in the formation of heterodimers in 50% of the dimerizations and the import of 50% of the truncated protein with the other 50% forming homodimers of truncated protein that are trapped in the cytosol. Taking into account some leakage during the homogenization process, this model accurately predicts the observed distribution of truncated thiolase.

To approach this question using the *in vivo* system it was necessary to demonstrate a physical interaction between subunits of dimers, some of which would be incompetent for import and others that would be competent. To this end a gene encoding epitope-tagged thiolase was constructed. The addition of the HA epitope tag makes an

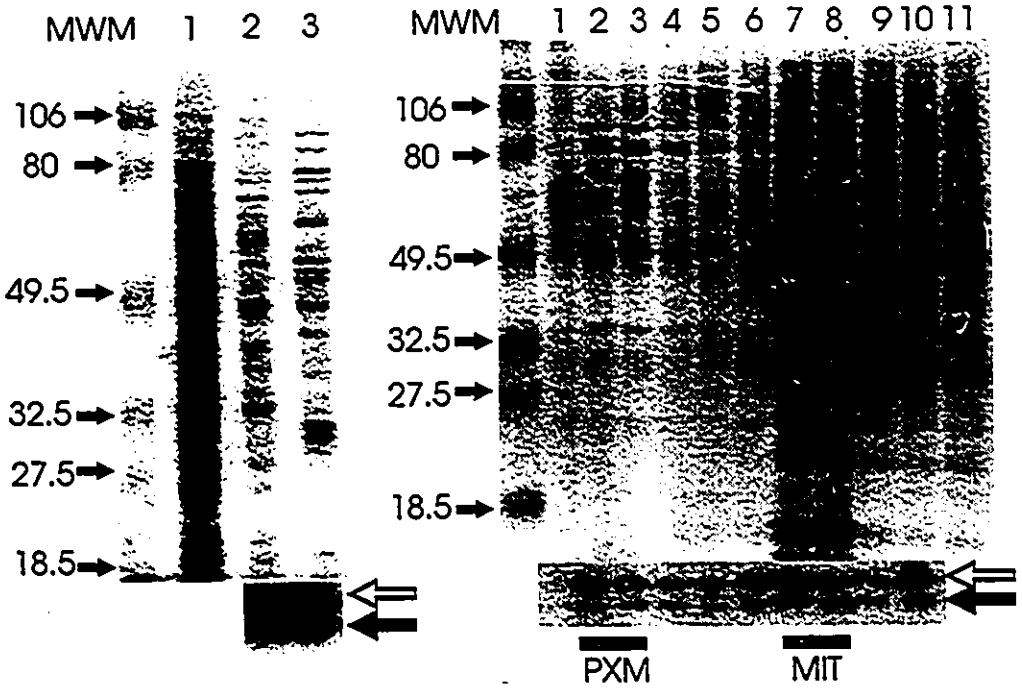

Figure 28. SDS-PAGE and western blot analysis of truncated thiolase co-expressed with the full-length protein in subcellular fractions. A 500 mL culture of DL1[SCTΔN] was grown for 18 h in SCIM. The postnuclear supernatant (Panel A, lane 1) was divided into a 20,000 x *g* supernatant (Panel A, lane 2) and 20,000 x *g* pellet (Panel A, lane 3). The pellet was further fractionated on a discontinuous Nycodenz™ gradient (Panel B). Equal portions of each fraction were resolved by SDS-PAGE on a 10% gel. Gels were stained with Coomassie blue. Western blots were probed with anti-*S. cerevisiae* thiolase serum followed by ^{125}I-Protein A. Open arrow = full-length thiolase. Filled arrow = truncated thiolase. M_r of standards are given in kDa. PXM = fractions enriched for peroxisomes. MIT = fractions enriched for mitochondria.

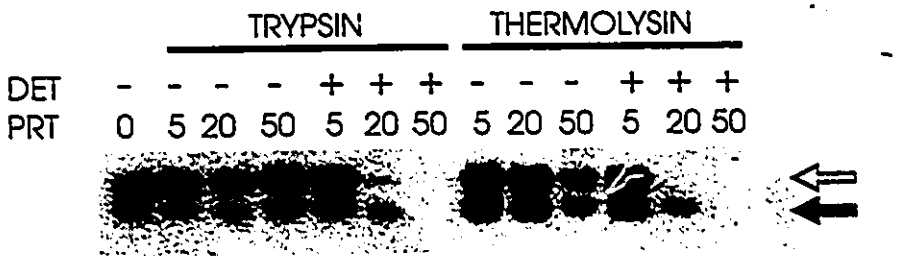

Figure 29. Western blot analysis of protease sensitivity of full-length and truncated thiolase in the organellar pellet. 500 μg of protein from the 20,000 x *g* pellet of DL1[ΔN] was treated with 2, 10, or 50 μg of either trypsin or thermolysin (PRT) in the absence or presence of 1% Triton X-100 and 1% deoxycholate (DET) for 15 min on ice. 10% of each reaction was resolved by SDS-PAGE on a 10% gel. The proteins were transferred to nitrocellulose and the blots were probed with anti-*S. cerevisiae* thiolase serum followed by ^{125}I-Protein A. The immunoreactive bands were visualized by exposure to X-ray film at -70°C with an intensifying screen for 72 h.

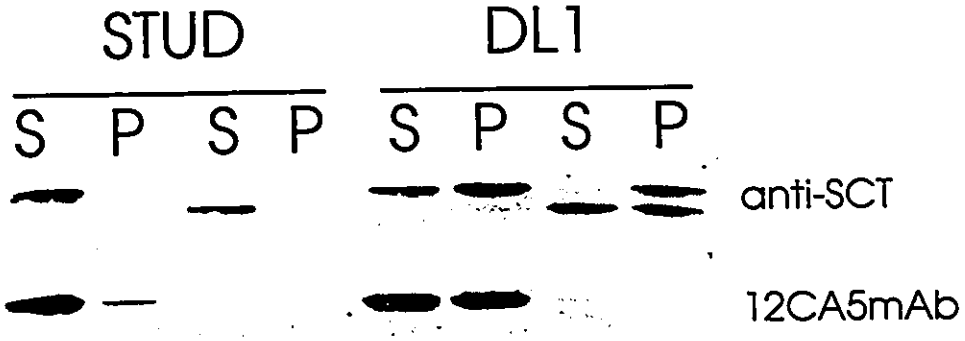

Figure 30. Western blot analysis of subcellular fractions of truncated and HA epitope-tagged thiolase expressed in the presence and absence of the full-length protein. 10 mL cultures of STUD[ΔN], DL1[ΔN], STUD[HA], and DL1[HA] were grown for 18 h. Equivalent proportions of 20,000 x *g* supernatant (SUP) and 20,000 x *g* pellet (PEL) were resolved by SDS-PAGE on duplicate 10% gels. Proteins were transferred onto nitrocellulose. One blot was probed with anti-*S. cerevisiae* thiolase serum followed by AP-conjugated anti-rabbit IgG. The duplicate blot was probed with 12CA5mAb followed by AP-conjugated anti-mouse IgG. Immunoreactive bands were visualized with NBT/BCIP.

immunologically distinct thiolase that can be specifically identified on western blots and immunoprecipitated with the 12CA5mAb (Kolodziej and Young, 1991).

A synthetic oligonucleotide duplex was inserted into the *Nco*I site of the plasmid encoding the truncated form of thiolase (see Fig. 2, pSCTΔN). The oligonucleotides were designed such that insertion of one or more oligonucleotide duplex could result in an epitope-tagged thiolase subunit with one or more copies of the tag located at the N-terminus of the protein. One of the clones recovered from the ligation experiment encoded two copies of the tag. The protein expressed from this gene was designated SCT(HA)2. Preliminary experiments indicated that SCT(HA)2 exhibited exactly the same subcellular distribution as the truncated thiolase when expressed in the presence or absence of the full-length protein (Fig. 30). In a protease protection experiment in which the organellar pellet of strain DL1[HA] was digested with trypsin in the absence and presence of 1% Triton X-100 only limited proteolysis was observed (Fig. 31). The tryptic digestion products of both thiolase molecules migrated as a single band with a subunit mass close in size to the truncated form of thiolase (SCTΔN). Western blot analysis of the same fractions with 12CA5mAb indicated that the lower molecular weight form had no HA epitope tag which constitutes the N-terminus of the tagged monomer. The most likely trypsin sensitive site common to both the full-length and epitope-tagged proteins is the Lys-Arg-Lys cluster at amino acid positions 23 to 25 in the wild type protein. Although a more detailed analysis is required, it seems likely that the bulk of thiolase forms a tightly folded protease-resistant structure in the native enzyme. In

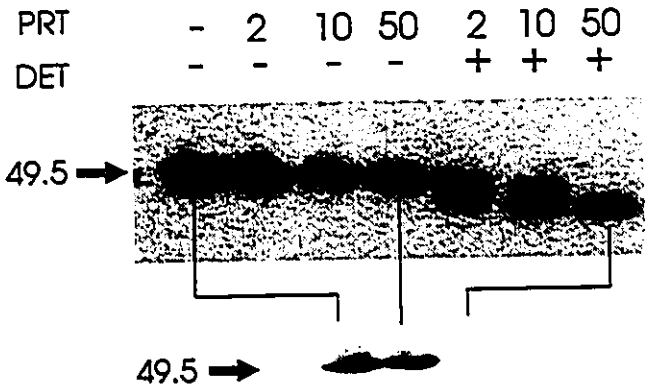

Figure 31. Limited proteolysis of thiolase. A 20,000 × *g* pellet was prepared from a 10 mL culture of DL1[HA]. Aliquots containing 60 µg of protein from this pellet were incubated on ice for 15 min with (+) or without (-) 1% Triton X-100 (DET) and then digested for 20 min with 2, 10 or 50 µg of trypsin (PRT). Digestion was terminated with the addition of hot SDS-PAGE buffer. Proteins were resolved by SDS-PAGE on a 7 to 15% gradient gel. The proteins were transferred to nitrocellulose and the blot was probed with anti-thiolase serum followed by HRP-conjugated anti-rabbit IgG. Immunoreactive bands were detected by ECL™. Selected digests were analyzed by western blotting with 12CA5mAb followed by AP-conjugated anti-mouse IgG. Immunoreactive bands were visualized with NBT/BCIP.

contrast, the N-terminus, either epitope-tagged or with the PTS2 of the full-length protein, is exposed in a protease-sensitive manner. Such a conformation would suggest that the targeting signal of thiolase is exposed, even though the protein is fully folded and dimerized in the cytosol, thus permitting the folded protein to be engaged by the putative PTS2 receptor.

The simultaneous expression of truncated and epitope-tagged thiolase with the full-length thiolase can be used to distinguish between two possible modes of translocation. One model is that both dimer subunits are engaged simultaneously at the cytosolic surface of the peroxisome but are translocated independently. Although there is randomized mixture of dimers created in the cytosol, only a select, non-random population of dimers interact with peroxisomes (*ie.* only heterodimers containing at least one targeting signal). Independently translocated subunits released into the lumen of the peroxisome would be free to dimerize with any other available monomer leading to the re-establishment of a randomly mixed population, including some heterodimers composed of subunits lacking the targeting signal. On the other hand, if dimer subunits remain associated throughout the translocation, only heterodimers with at least one targeting signal will be found in the imported fraction.

As a prelude to this analysis, the characteristics of strains expressing all three thiolases were examined in greater detail. In Fig. 32 analysis of the subcellular distribution of thiolases is analyzed in a strain expressing moderate (DL1[ΔN+HA]) and 5- to 7-fold higher (DL1[ΔN+HA]^{HC}) levels of the untargeted subunits along with the full

length protein. In (DL1[ΔN+HA]^{HC}) untargeted subunits are expressed at such high levels relative to the full-length protein (HIGH, lane 1) that virtually all full-length subunits may be expected to form heterodimers with one or another of the cytosolic variants. Thus the total amount of epitope-tagged and truncated subunits translocated into the pellet was equal to the amount of full-length thiolase in the pellet (HIGH, lane 3). First, this observation indicates that the translocation of cytosolic subunits is efficient. If untargeted subunits were less efficiently translocated than the full-length thiolase, the combined total of the untargeted subunits would be discernably less than that of the full-length protein. Secondly, the ratio of translocated epitope-tagged to truncated thiolase is consistent with the availability of these subunits in the cytosol during dimer formation (HIGH, lane 1 shows that the epitope-tagged form of thiolase is somewhat more abundant than the truncated subunit in the total and is therefore also more abundant in the translocated fraction lane 3). A preference for heterodimerization between the full-length thiolase and one of the variant forms would skew the distribution of subunits in the translocated fraction in favour of the preferred subunit.

In a previous section the disassembly of peroxisomes was examined (see Fig. 15), and full-length thiolase was found to be only partially solubilized by mild treatment with detergent. Treatment of the organellar fraction from DL1[ΔN+HA] and DL1[ΔN+HA]^{HC} with 1% Triton X-100 results in solubilization only a portion of the thiolases (Fig. 32, lanes 4 and 5, 12 and 13) in a manner consistent with that observed for wild type thiolase alone. The anti-SKL reactive proteins of 92 kDa (multifunctional protein) and 54 kDa

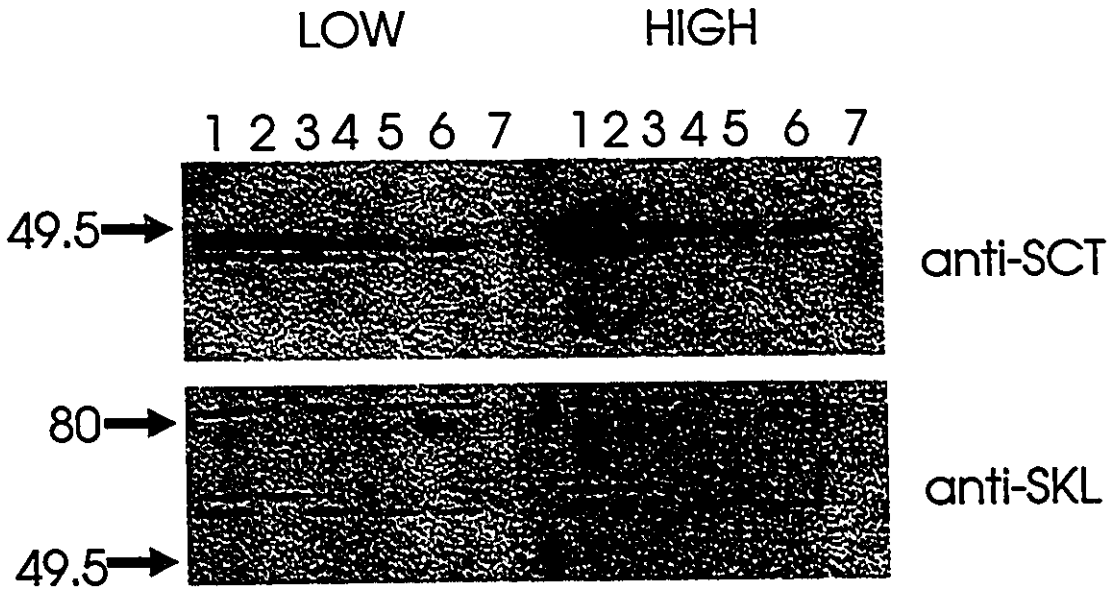

FIG. 32. Western blot analysis of truncated and HA epitope tagged thiolase expressed simultaneously with the full length protein from low or high copy number vectors. 10 mL cultures of DL1[ΔN+HA] (LOW) and DL1[ΔN+HA]^{hc} (HIGH) were grown for 18 h in SCIM. The post nuclear supernatants (lane 1) were divided into 20,000 X *g* supernatant (lane 2) and pellet (lane 3). Samples of the pellet were treated with 1% TX-100 on ice for 30 min and centrifuged at 20,000 X *g* for 20 min to generate a soluble (lane 4) and particulate fraction (lane 5). Another sample of pellet was treated with 10 mM Tris-Hcl pH 8.5, 500 mM NaCl, and 2.5 mM EDTA for 30 min on ice and centrifuged for 30 min at 100,000 X *g* to generate a soluble (lane 6) and particulate (lane 7) fraction. Proteins were resolved by SDS-PAGE on duplicate 7 to 15% gels and transferred to NC. One blot was probed with anti-SCT antiserum diluted 1:5000. The duplicate blot was probed with anti-SKL antiserum diluted 1:200. The immunoreactive bands were detected with ^{125}I-Protein A and exposure on a phosphor storage screen.

(peroxisomal citrate synthase isoenzyme) are not solubilized at all by detergent, which may indicate that they associate more strongly with a detergent resistant protein "core" structure similar to that seen in rat and mouse peroxisomes (Poole and Crane, 1992; Hayashi *et al.*, 1981; Thompson and Krisans, 1990; Alexson *et al.*, 1985). Both thiolases, and anti-SKL-reactive proteins are released by treatment with 10 mM Tris-HCl pH 8.5, 2.5 mM EDTA, 0.5 M NaCl (Fig. 32, lanes 6 and 7, 14 and 15) which disrupts the peroxisomal membrane and probably interactions among elements of the peroxisomal matrix. The significance of this observation is that heterodimers of the full-length and truncated or epitope-tagged thiolase subunits appear to assemble into peroxisomes in the same way as homodimers of the full-length protein and are equally susceptible to the partial or complete disassembly of the organelle. In addition the import of cytosolic subunits does not interfere with the import of at least two other matrix proteins.

To directly demonstrate dimer interactions, cells of strain DL1[ΔN+HA] were labelled with ^{35}S-methionine for 15 min, chased for 20 min, spheroplasted, homogenized and divided into cytosolic (SUP) and organellar (PEL) fractions. These fractions were solubilized with 50 mM Tris-HCl pH 8.5, 2.5 mM EDTA, 500 mM NaCl for 30 min. This treatment is better than detergent for complete solubilization of peroxisome matrix elements (see Fig. 32). The solubilized thiolase dimers were then immunoprecipitated with anti-*S. cerevisiae* thiolase serum and 12CA5mAb (Fig. 33). Immunoprecipitates obtained with anti-*S. cerevisiae* thiolase serum represent the total labelled thiolase profile in each fraction. The immunoprecipitates obtained with 12CA5mAb should contain the

epitope-tagged protein and presumably any subunits with which it is dimerized.

The results indicate that epitope-tagged/truncated thiolase dimers are found exclusively in the cytosolic fraction while epitope-tagged/full-length dimers are present in the organellar fraction (Fig. 33, lanes 3 and 4). A large amount of epitope-tagged protein was immunoprecipitated from the cytosol because in addition to heterodimers with the truncated protein, import incompetent homodimers of epitope-tagged protein and heterodimers of epitope-tagged/full length protein that leak during homogenization or have not yet been imported are also present in the cytosolic fraction.

A generally accepted model of protein translocation envisions a the translocating peptide within the hydrophillic pore created by components of the translocon as having some secondary structure but no tertiary or quaternary interactions (except transiently with surfaces within the pore). A model of thiolase translocation in which dimers are simultaneously committed to translocation but are actually threaded through the translocon consecutively and independently would be consistent with the accepted model. Monomers would then be released from the translocon into the lumen of the peroxisome for refolding and oligomerization. However, no dimers between the epitope-tagged and truncated thiolase that are predicted by this model were detectable in immunoprecipitates from the organellar fraction.

An alternative relatively complex model that can explain these results is one in which subunits of heterodimers are translocated independently with the leading subunit remaining in contact with the lumenal surface of the translocon until the trailing subunit

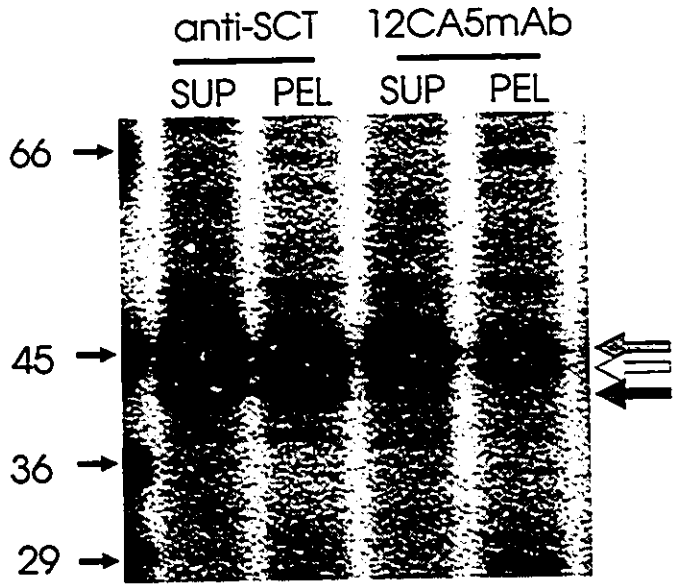

Figure 33. Immunoprecipitation of thiolase from subcellular fractions of ^{35}S-labelled yeast expressing truncated, HA-epitope-tagged and full-length thiolase. $5 \cdot 10^8$ cells of YNBD-grown DL1[HA+ΔN] were transferred into YNO and grown for an additional 6 h for the induction of peroxisomes. The cells were concentrated by centrifugation and labelled for 15 min at 30°C with 500 µCi of ^{35}S-methionine in 1 mL YNO. The cells were concentrated and resuspended in 10 mL YNO with 20 mM unlabelled methionine. The labelled cells were fractionated into 20,000 x *g* supernatant (SUP) and 20,000 x *g* pellet (PEL) fractions. The fractions were adjusted to 50mM Tris-HCl pH 8.5, 2.5 mM EDTA, 500 mM NaCl and incubated on ice for 30 min. The solubilized fractions were centrifuged at 100,000 x *g* for 20 min and the supernatants were split. Thiolases were immunoprecipitated with either 2.5 µL anti-*S. cerevisiae* thiolase serum or 10 µL of 12CA5 ascites fluid at room temperature for 1 h. Immune complexes were adsorbed to Pansorbin™ for 1 h at room temperature. The pelleted cells were washed 4 times with Tris-HCl pH 8.5, 2.5 mM EDTA, 500 mM NaCl. The final pellet was suspended in SDS-PAGE sample buffer, boiled and centrifuged. 33 % of each supernatant was resolved by SDS-PAGE on a 7 to 15% gradient gel. The gel was dried and exposed on a phosphor storage screen. Open arrow=full-length thiolase. Filled arrow = truncated thiolase. Grey arrow = HA epitope-tagged thiolase.

is translocated. The dimers released into the peroxisomal matrix would have the same subunit composition as those arriving at the cytosolic surface of the translocon.

The simpler explanation for the observed results is that dimers are translocated simultaneously and remain in contact during the import process. Since nothing is currently known about the structural requirements for dimerization of thiolase monomers it is possible to speculate that the bulk of the protein may be unfolded without disrupting the interface between monomers. Nonetheless, in order for monomers to remain associated throughout the translocation event, at least some portion of both monomers containing the dimer interface must occupy the channel of the translocon simultaneously. Oligomerization of glyoxysomal malate synthase octamers (Kruse and Kindl, 1983) and *C. boidinii* alcohol oxidase octamers (Goodman *et al.*, 1984) appears to occur only after the monomeric subunits have been synthesized in the cytosol and translocated into the organelle. When complementary Zellweger fibroblasts are fused, 3-aminotriazole which covalently modifies catalase and stabilizes its folded structure, inhibits the redistribution of cytosolic catalase to peroxisomes relative to untreated heterokaryons (Middlekoop *et al.*,1991). These examples support the idea that peroxisomes do not routinely import folded, oligomerized proteins.

In contrast, purified octameric alcohol oxidase of *Pichia pastoris* appeared to be quickly incorporated in to punctate structures after microinjection into intact mammalian cells some, but not all of which, co-stained with anti catalase antibodies (Walton *et al.*, 1992a). Likewise, fusion of certain complementary Zellweger fibroblasts results in the

very rapid assembly of pre-existing, active catalase (ie. heme-containing tetramers) into peroxisomes (Brul *et al.*, 1988). Although there is no direct evidence that the rapid import of these complexes does not involve, at some point, the disassembly and unfolding of subunits which are then targeted independently, the possibility of the co-translocation of preassembled subunits cannot be ruled out either.

Interestingly, the translocation of human serum albumin decorated with several covalently attached peptides of 12 amino acid residues ending in SKL-COOH, has been observed in microinjected (Walton *et al.*, 1992b) and streptolysin O-permeabilized (Wendland and Subramani, 1993a) mammalian cells. The aspect of this observation which is pertinent to modelling thiolase translocation is that at least two polypeptide segments are obliged to occupy the channel simultaneously during the translocation of these "branched" molecules.

Several lines of evidence make it clear that the post-translational translocation of proteins is sensitive to the conformation of the translocating species. The first is that fully folded precursors are poorly imported *in vivo* without prior treatment with chaotropic reagents such as urea (Eilers *et al.*, 1988). If the unfolded polypeptide is rapidly diluted into the import mixture, translocation can be observed. Mitochondrial precursor and mature proteins undergo a partial unfolding when bound to the surface of isolated mitochondria (Eilers *et al.*, 1988; Endo *et al.*, 1989; Hartmann *et al.*, 1993). Mutations which inhibit oligomerization can also apparently improve the efficiency of import (Nye and Scarpulla, 1990; Chen and Douglas, 1988). The introduction of point mutations

which destabilize the folded structure of a passenger protein domain enhances its post-translational import (Vestweber and Schatz, 1988a).

Likewise, treatments which constrain the conformation of the protein are inhibitory to import. The binding of the folate analogue methotrexate to DHFR fused to a presequence inhibits post-translational import of the fusion protein into mitochondria (Eilers and Schatz, 1986). *In vivo* import of a metal-chelating protein (copper metallothionein), targeted *via* a presequence to yeast mitochondria was inhibited in media containing copper (Chen and Douglas, 1987; Nye and Scarpulla, 1990) presumably because multiple amino acid side chains co-ordinating metal ions stabilize the metal-bound conformation of the protein. A protein domain whose structure is stabilized by internal disulfide bonds becomes imbedded in mitochondrial import sites but is not translocated (Vestweber and Schatz, 1988b).

Finally, molecular chaperonins can function in the import pathway at several points (Rothman, 1989; Hendrick and Hartl, 1993; Craig *et al.*, 1990). Chaperones can apparently interact directly with proteins destined for translocation and maintain them in an import competent conformation. Members of the 70 kDa heat shock protein family have been implicated in translocation into endoplasmic reticulum (Deschaies *et al.*, 1988; Zimmerman *et al.*, 1988), mitochondria (Murakami *et al.*, 1988,1990; Sheffield *et al.*, 1990), chloroplasts (Waegmann *et al.*, 1990), and nuclei (Shi and Thomas, 1992). Cuezva *et al.* (1993) have suggested that chaperones may also participate in the import of peroxisomal proteins. In mammalian cells, peroxisomal import is diminished in cells

which have been depleted of a 73 kDa hsp by pre-treatment with anti-hsp73 antibodies (Paul Walton, personal communication).

Compared to the other intracellular compartments, the study of peroxisomal translocation is at a formative stage. A possibility exists that the translocation of cytosolic variants of thiolase via dimerization represents a distinctive mode of translocation that is unique to peroxisomes. In order to determine if another, well characterized translocation apparatus could accommodate the translocation of thiolase dimers, I investigated the distribution of cytosolic thiolase in the peroxisome-deficient, thiolase-deficient strain YPHSTUD expressing the PTS2 mutant Q6R/H11Y, which is apparently targeted to mitochondria. This experiment indicated that a substantial amount of epitope-tagged thiolase co-migrates with Q6R/H11Y into the organellar fraction of this strain (Fig. 34, lane 8). Like peroxisomes, mitochondria can also accommodate the import of branched-chain molecules (Vestweber and Schatz, 1988c). It therefore seems unnecessary to ascribe unusual properties to the peroxisomal translocation machinery, and it is more likely that translocating thiolase dimers assume a conformation that can be accommodated by a "generic" translocon.

At the very least, the final set of experiments have disclosed a novel import pathway for a cytosolic version of thiolase *via* dimerization with a targeted monomer. The preliminary results indicating that dimerization with Q6R/H11Y can also confer import into mitochondria in the peroxisome-deficient strain YPHSTUD suggests that this property resides with thiolase and is not due to some distinctive feature of the import

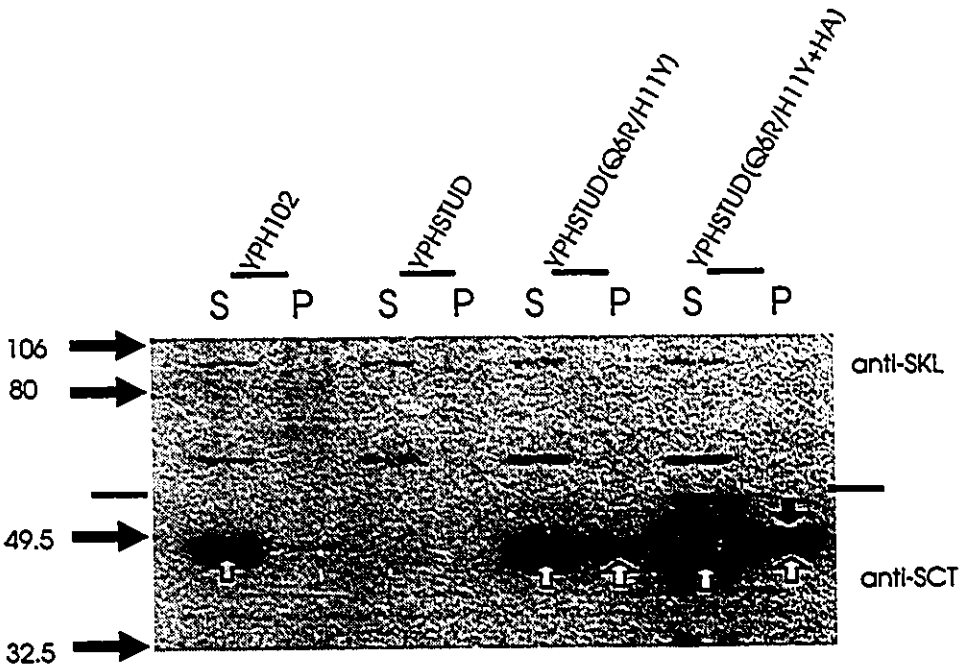

Figure 34. Western blot analysis of subcellular fractions of HA epitope-tagged thiolase coexpressed with a full-length thiolase redirected to mitochondria. 10 mL cultures of YPH102, YPHSTUD, YPHSTUD[H11Y], and YPHSTUD[H11Y+HA] were grown for 20 h in SCIM. Equivalent proportions of the 20,000 x g supernatant (S) and 20,000 x *g* pellet (P) were resolved by SDS-PAGE on a 7 to 15% gradient gel. The proteins were transferred to nitrocellulose and the blot was probed with anti-*S. cerevisiae* thiolase serum followed by HRP-conjugated anti-rabbit IgG. Immunoreactive bands were visualized with ECL™ with a 1 h exposure on X-ray film. Filled arrow = HA-tagged thiolase. Open arrow = Q6R/H11Y thiolase. Grey arrow = wild type thiolase.

apparatus of peroxisomes. More work will be required to determine the structure of the thiolase translocation intermediate and the nature of the translocon itself.

3.6. FUTURE DIRECTIONS

An important feature of peroxisomal protein targeting is the existence of at least two distinct classes of targeting signals with divergent translocation initiation events. The analysis of genes involved in various peroxisome assembly defects in yeast will likely identify many components which are required for peroxisomal protein import. The temperature-sensitive targeting mutants of thiolase identified in this work (L12S, for example) can be used to identify components of the import pathway specific to PTS2 pathway. When overexpressed from a library constructed in high copy-number plasmids, components involved in thiolase import may suppress the growth defect at 30°C by permitting the import of a minimal amount of mutant thiolase required to restore oleic acid metabolism.

With respect to dimer translocation, it would be of considerable interest to determine if this translocation mode exists for other proteins which exist as dimers in the native state. In *S. cerevisiae* the multifunctional enzyme, peroxisomal citrate synthase, and peroxisomal malate dehydrogenase are all dimers which are targeted by PTS1 motifs.

Several methods may be used to determine possible translocation-competent conformations of the yeast thiolase. For example, molecular sizing techniques such as native polyacrylamide gel electrophoresis or gel filtration could be used to simultaneously

measure changes in hydrodynamic radius of the molecule as dimers are unfolded and dissociated in the presence of different concentrations of chaotropic reagents such as urea or guanidine hydrochloride. A finding that the globular domain of the protein unfolds prior to the breakdown of the dimer interface would suggest that an intact dimer could traverse the membrane in a nearly completely unfolded state. Dr. David W. Andrews (Department of Biochemistry, McMaster University) has observed that many proteins in current data bases which feature significant hydrophobic stretches located at the C-terminus are dimers. Thiolase is one of these. If the dimerization interface of thiolase were restricted to this region and could function more or less independently of the structure of the bulk of the protein then the translocating dimer could assume a tail-to-tail configuration without requiring significant higher order structure except in a limited portion where the two subunits interact.

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