ACYL-COA SYNTHETASE IN *ESCHERICHIA COLI*

PURIFICATION AND CHARACTERIZATION OF ACYL-COA SYNTHETASE IN *ESCHERICHIA COLI*: RELATION TO FATTY ACID UPTAKE AND METABOLIC STATES OF THE CELLS

By

OSCAR CHENG, B.Sc. (University of Toronto)

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1997)
(Biochemistry)

McMaster University
Hamilton, Ontario

TITLE: Purification and Characterization of Acyl-CoA Synthetase in *Escherichia coli*: relation to fatty acid uptake and metabolic states of the cells

AUTHOR: Oscar Cheng, B.Sc. (University of Toronto)

SUPERVISOR: Professor G.E. Gerber

NUMBER OF PAGES: xiii, 81

ABSTRACT

The Gram-negative bacterium *Escherichia coli* can live on long chain fatty acids as its sole carbon source. However, the rate of fatty acid uptake was reduced after starvation (Mangroo, 1992). In this study, it was found that this starvation effect of reducing oleate uptake rate could only be observed in the initial minute post starvation. The starvation effect was not due to the depletion of its substrates ATP or reduced coenzyme A. Neither was the effect caused by a lowered acyl-CoA synthetase level. This reduction in oleate uptake rate can be reversed by incubation in oleate.

It has been reported that lactate activated oleate uptake in *Escherichia coli* (Mangroo and Gerber, 1993). In the present study, it was discovered that when [9,10-^{3}H]oleate was used as the radioactive tracer in uptake assay, the majority of the radioactivity effluxed from the cells. It was found that lactate did not have an effect on the overall oleate uptake. Its apparent activation on oleate uptake was due to the reduction in the efflux rate of radioactive probes, instead of increasing the uptake rate of oleate.

Acyl-CoA synthetase in *Escherichia coli* has always been described as a protein of around 45 kDa (Kameda and Nunn, 1981; Kameda *et al.*, 1986). However, the *fadD* gene that encodes the *Escherichia coli* acyl-CoA synthetase predicts a 62 kDa protein (Black *et al.*, 1992; Fulda *et al.*, 1994). This new form of acyl-CoA synthetase has been partially purified. This form, which has a size

of around 62 kDa, can be converted to its 45 kDa counterpart in a Triton X-100 and temperature dependent manner. This induced processing of the 62 kDa enzyme was found to be inhibited by oleate, a natural substrate of acyl-CoA synthetase. The two forms of acyl-CoA synthetase have also been shown to be immunologically related.

A much simpler purification scheme was developed for the 45 kDa acyl-CoA synthetase using the membrane bound form of acyl-CoA synthetase. The selective extraction of the 62 kDa acyl-CoA synthetase from the membrane, together with the induced processing into the 45 kDa form has provided the basis for this comparatively simple process. The availability of the expression system made the low yield associated with this simple process acceptable.

Using oleate as substrate, the K_M and V_{max} for the 45 kDa acyl-CoA synthetase were determined to be 85 $\pm$ 18 μM and 1550 $\pm$ 165 nmole/min/mg protein respectively, whereas the K_M and V_{max} for the 62 kDa enzyme were determined to be 38 $\pm$ 12 μM and 633 $\pm$ 79 nmole/min/mg protein respectively. This suggested that the 62 kDa enzyme has higher affinity towards oleate than its 45 kDa counterpart under standard assay conditions while the ratio of V_{max}/K_M remained relatively constant.

Evidence suggesting Triton X-100 activates acyl-CoA synthetase by providing a surface for catalysis was presented. This interaction between acyl-

CoA synthetase and the Triton X-100 / oleate mixed micelle could be affected by the surface charge of the mixed micelle.

ACKNOWLEDGEMENTS

I would like to thank Professor G.E. Gerber for his inspiration, guidance, encouragement, support, and his belief in my abilities. I would like to thank the members of my supervisory committee, Professors R. Epand and G. Wright for their guidance and helpful discussions.

I would like to thank Dr. T. Schmelter for his guidance, patience and most importantly for his encouragement. I would also like to thank my co-workers Jae-Ho Yoo and Scott Covey for their helpful discussions. I would like to thank Tom Galt for his excellent technical assistance.

Lastly, I wish to acknowledge the financial support of McMaster University.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
Brij	polyoxyethylene 20 cetyl ether
BSA	bovine serum albumin
°C	degrees, Celsius
Ci	curie
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetate
fad	fatty acid degradative
$FADH_2$	reduced flavin adenine dinucleotide
×g	acceleration of gravity
g	gram
hr	hour
Ig	immunoglobulin
IPTG	isopropyl β-D thiogalactopyranoside
kDa	kiloDalton

K_M	Michealis constant
l	liter
μ	micro-
m	milli-
M	molar
min	minute(s)
mol	moles
n	nano-
NAD	nicotinamide adenine dinucleotide
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
S.D.	standard deviation
SDS	sodium dodecylsulphate
sec	second(s)
tris	tris-(hydroxymethyl)aminomethane
V_{max}	maximal velocity

1. INTRODUCTION

The Gram-negative bacterium *Escherichia coli* can live on long chain fatty acids as its sole carbon source. For this to happen, it must have mechanisms for the uptake of long chain fatty acids, to harness the energy stored in these compounds, and to be able to break down these 12 to 20 carbon containing compounds into smaller building blocks for growth. The uptake of fatty acids requires at least two proteins: the Fad L facilitates fatty acid transverse of the outer membrane, whereas the Fad D (acyl-CoA synthetase) converts the fatty acid into an acyl-CoA thioester in the cytoplasm. The activated fatty acid cannot permeate the inner membrane, hence it is trapped inside the cytoplasm. In addition, it is through this activated form of fatty acid that further reactions occur to harness the energy stored in fatty acid and to form building blocks for growth. Therefore, it is the main goal of this thesis to understand the properties of acyl-CoA synthetase and its relationships to fatty acid uptake at different metabolic states of *Escherichia coli*.

1.1 Membrane structure of *Escherichia coli*

Escherichia coli, like other Gram-negative bacteria, has a double membrane system. The outer membrane is impermeable to hydrophobic substances. This may be due to its high content of lipopolysaccharides, a hydrophilic substance (Nikaido and Vaara, 1985). The peptidoglycan layer lies

beneath the outer membrane and within the periplasmic space. This layer is composed of saccharides and proteins, and gives the bacteria its rigidity (Nikaido and Vaara, 1985). The periplasmic space contains proteins that are responsible for the detection, processing, and transport of essential nutrients (Oliver, 1996). The inner membrane, also known as cytoplasmic membrane, is similar to membranes in eukaryotic cells, both structurally and functionally (Kadner, 1996). It contains 65 to 75% of the total cellular phospholipids, and 6 to 9% of the cellular proteins (Kadner, 1996).

1.2 Fatty acid uptake in *Escherichia coli*

Since the outer membrane of *Escherichia coli* is impermeable to hydrophobic compounds, the permeation of long chain fatty acids across the outer membrane is facilitated by the product of the *fadL* gene. Once long chain fatty acids get across the outer membrane, they are believed to transverse the periplasmic space and the inner membrane by diffusion (Mangroo and Gerber, 1992).

The next step involves acyl-CoA synthetase (fatty acid : CoA ligase EC 6.2.1.3.), which converts the long chain fatty acids into the fatty acyl-CoA thioesters. This requires the input of one molecule each of ATP and CoA per reaction. The thioester is hydrophilic enough, such that efflux across the inner membrane is insignificant (Maloy *et al.*, 1981). This helps the build up of the fatty acyl-CoA thioesters pool in the cytoplasm. It is in this manner that acyl-CoA

synthetase is involved in the uptake of long chain fatty acids, and the process has been termed vectorial acylation (Klein *et al.*, 1971). Acyl-CoA thioester can be catabolised further or it can be used directly for phospholipids biosynthesis (Black and DiRusso, 1994).

It has been shown that the rate of fatty acid uptake was reduced after starvation (Mangroo, 1992). This reduction in fatty acid uptake rate could be reversed by incubating the starved cells in lactate prior to the fatty acid uptake assay (Mangroo, 1992). It was also shown that this reduction in uptake rate was not due to depletion of ATP, nor was it caused by a diminished membrane potential (Mangroo, 1992).

1.3 Fatty acid oxidation

To harness the energy stored in long chain fatty acids, and to break them down into smaller building blocks, the long chain fatty acids have to be activated before hand. This is achieved by acyl-CoA synthetase in the process of vectorial acylation. Fatty acyl-CoA thioester, the activated form of fatty acid, can then be fed into β-oxidation cycle. The minimal requirements of the β-oxidation cycle are the activities of the enzymes acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (Nunn, 1986; Figure 1). A two carbon unit in the form of acetyl-CoA is cleaved

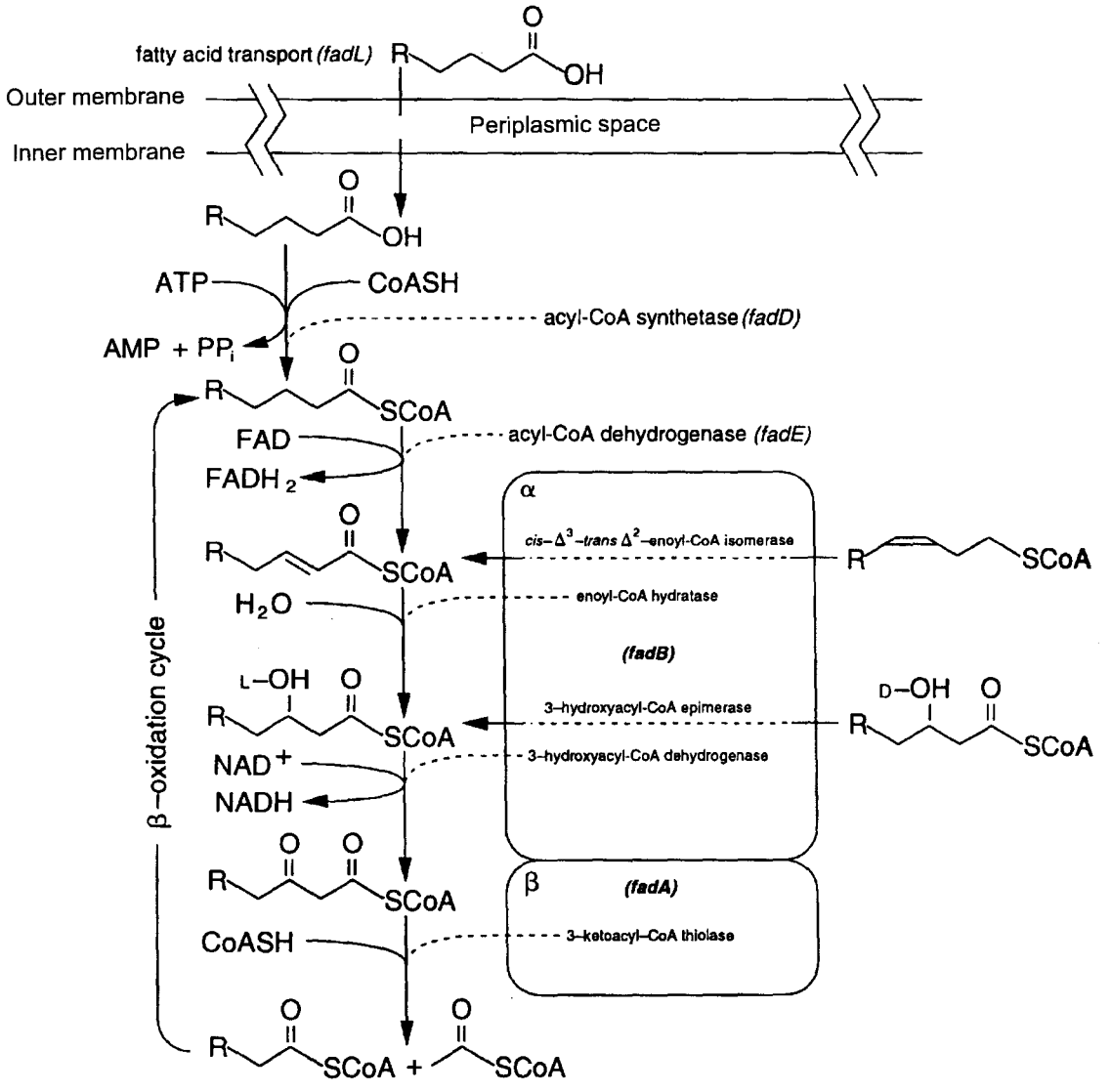

Figure 1. Schematic representation of β-oxidation. Adapted from Clark and Cronan (1996).

off from the fatty acyl-CoA thioester during each turn of the cycle. At the same time, one molecule each of NADH and $FADH_2$ is produced. Acetyl-CoA can enter the tricarboxylic acid cycle for generation of more NADH and $FADH_2$. This stored reducing power is then fed into the electron transport chain for ATP synthesis.

1.4 Glyoxylate bypass

For *Escherichia coli* to grow using fatty acids as sole carbon source, the bacteria must be able to synthesize complex molecules from fatty acids. This is achieved by first breaking fatty acids down into acetyl-CoA. By means of tricarboxylic acid cycle, it can generate the intermediates, e.g. amino acids, for biosynthesis. While each turn of the tricarboxylic acid cycle has one molecule of acetate equivalent (2 carbon unit) as input, it also releases two molecules of carbon dioxide. Therefore, the tricarboxylic acid cycle cannot have a net anabolic effect, by itself, on biosynthesis. The glyoxylate shunt solves this problem by providing a by-pass of those steps that release carbon dioxide in the tricarboxylic acid cycle (Cronan and LaPorte, 1996; Figure 2).

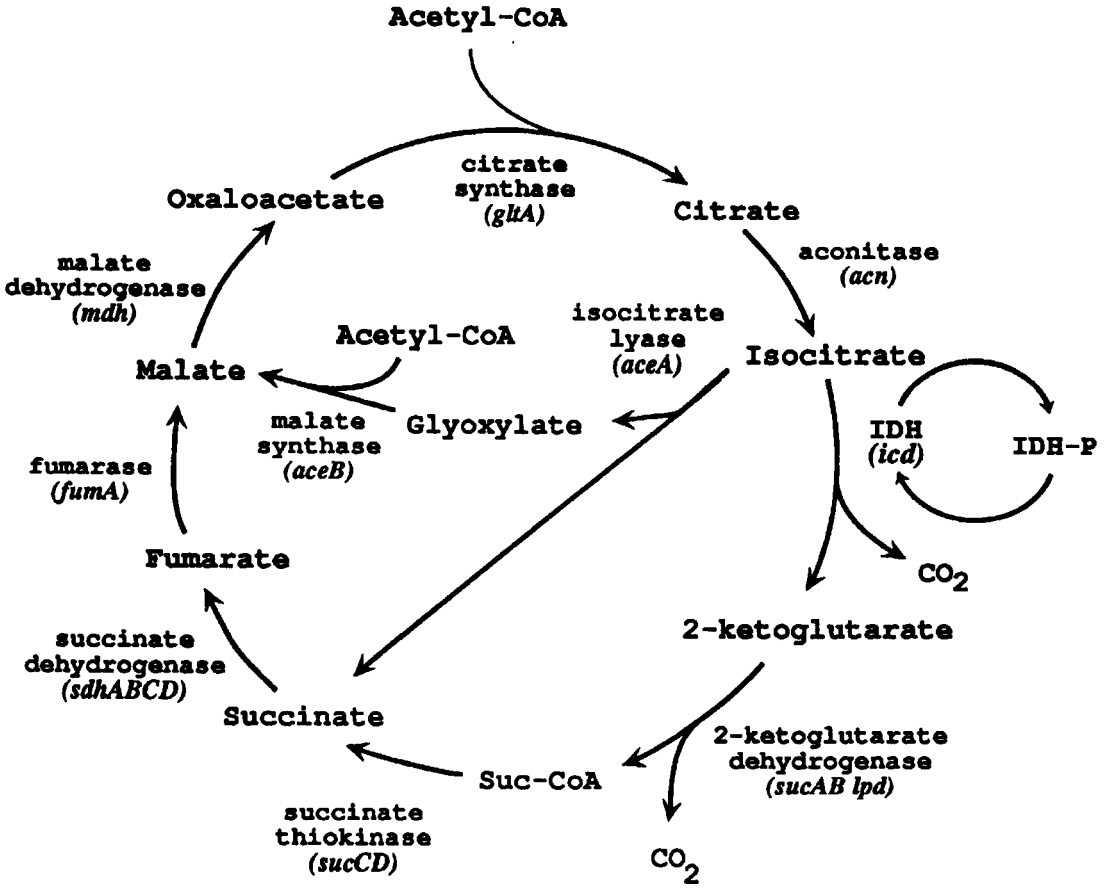

Figure 2. Schematic representation of the tricarboxylic acid cycle and the glyoxylate bypass. Taken directly from Cronan and LaPorte (1996).

1.5 Coordinated expression of enzymes involved in transport, activation, and oxidation of fatty acids

Fatty acids are considered to be "low status" energy sources by *Escherichia coli* (Clark and Cronan, 1996). Therefore, in the presence of other preferred energy sources, e.g., glucose, the expression of the enzymes required for the transport, activation and oxidation of fatty acids are repressed (Clark and Cronan, 1996). However, if fatty acid is the sole energy source, the ability of *Escherichia coli* to express those enzymes required for the metabolism of fatty acid will be essential for its survival. Therefore, when *Escherichia coli* is grown in medium where fatty acid is the only carbon source, the expressions of these enzymes are induced (Nunn, 1986). This regulation is controlled by the *fad* (fatty acid degradative) regulon. The *fad* regulon consists of the genes that encode for at least five different proteins (Clark and Cronan, 1996, Figure 1).
These genes, which are scattered around the chromosome, are repressed by the product of the *fadR* gene (Clark and Cronan, 1996).

It was found that the induced expression of *fad* enzymes requires the presence of fatty acids that are at least 12 carbons long (Overath *et al.*, 1969). It was also found that acyl-CoA synthetase is required for the induction, as mutants lacking acyl-CoA synthetase activity failed to respond to the long chain fatty acids present in the growth medium (Overath *et al.*, 1969). DiRusso *et al.* have

shown that the product of the *fadR* gene was inhibited from binding DNA by long chain fatty acyl-CoA thioesters, suggesting that long chain fatty acyl-CoA thioester is the effector molecule for the induction of the *fad* system (DiRusso *et al.*, 1992). In other words, the transcription of the *fad* genes is usually repressed by *FadR* binding to the genomic DNA, unless this binding of *FadR* is inhibited by the presence of long chain fatty acid-CoA thioesters.

1.6 Acyl-CoA synthetase

Acyl-CoA synthetase in *Escherichia coli* was first described in 1969 by Overath *et al.*. It catalyses the conversion of fatty acid to the corresponding fatty acyl-CoA thioester. This reaction requires the presence of ATP, CoA, fatty acid, and magnesium chloride (Overath *et al.* 1969). The size of the partially purified native enzyme was reported to be 120,000 kDa on Sephadex G-200 (Samuel *et al.*, 1970). Kameda and Nunn have similar observation on the size of acyl-CoA synthetase (130,000 kDa) that has been purified to homogeneity. In addition, the subunit size of the enzyme was determined to be 47 kDa by SDS-PAGE (Kameda and Nunn, 1981). However, in a 1985 study, the mobility of the purified acyl-CoA synthetase on SDS-PAGE was reported to be consistent with a 45 kDa protein (Kameda *et al.*, 1985). Further, the native enzyme in the above study behaved as a 45 kDa protein on Sephadex G-100 chromatography (Kameda *et al.*, 1985).

The *fadD* gene encoding acyl-CoA synthetase in *Escherichia coli* has been cloned (Black *et al.*, 1992; Fulda *et al.*, 1994). The gene encodes a predicted 62 kDa protein (Black *et al.*, 1992). Black *et al.* suggested that there is no evidence for the processing of the 62 kDa acyl-CoA synthetase in *Escherichia coli*. In an independent cloning studies, a conflicting *fadD* sequence was presented (Fulda *et al.*, 1994). Nonetheless, it also predicted the acyl-CoA synthetase has a size of 62.3 kDa (Fulda *et al.*, 1994).

1.7 Objectives and rationale

The finding that *Escherichia coli* reduces the uptake of a nutrient during starvation is unexpected. The finding that the bacteria would increase its fatty acid uptake in the presence of an alternate energy source, e.g., lactate, is equally as surprising. The above findings suggest that there may be some underlying reasons for the bacteria to go against the selective pressure of evolution. It is then the objective of the present study to pinpoint the exact cause(s) of both the starvation effect on the reduction of fatty acid uptake, and the lactate effect on the activation of fatty acid uptake.

It has been established that the formation of acyl-CoA thioester catalyzed by acyl-CoA synthetase is the rate limiting step in fatty acid uptake (Maloy *et al.*, 1981; O'Brien and Frerman, 1980). It follows that the depletion of any of the substrates for acyl-CoA synthetase during starvation would be an obvious cause for the observed reduction in uptake rate. Also possible is the decrease in level

of functional acyl-CoA synthetase. Therefore, monitoring the levels of the above mentioned factors during starvation served as a starting point for the present study.

Acyl-CoA synthetase in *Escherichia coli* has always been described as a protein of around 45 kDa on SDS-PAGE (Kameda and Nunn, 1981; Kameda *et al.*, 1986). However, the *fadD* gene that encodes the *Escherichia coli* acyl-CoA synthetase predicts a 62 kDa protein (Black *et al.*, 1992; Fulda *et al.*, 1994). Moreover, there is no evidence of a signal sequence or N-terminal amino acid modification in the enzyme which suggests post-translational processing (Black *et al.*, 1992). Therefore, it would be interesting to investigate the properties of this novel form of acyl-CoA synthetase *in vitro*, and to compare the findings with that of the 45 kDa form. This requires the purification of the 62 kDa acyl-CoA synthetase. On the other hand, it has been well established that the 45 kDa acyl-CoA synthetase is functional (Kameda and Nunn, 1981). This smaller form of the enzyme may also be in used *in vivo*. Therefore, a simple purification procedure will benefit the structural studies of the enzyme. Although purification methods have been described in the literature (Kameda and Nunn, 1981; Kameda *et al.*, 1986), these methods are complicated and lengthy, rendering them inappropriate for large scale preparation of acyl-CoA synthetase required for future structural analysis by e.g., X-ray crystallography. Another interesting aspect concerning acyl-CoA synthetase is whether the two forms of the enzyme

are related. Specifically, can the 62 kDa acyl-CoA synthetase be converted to its 45 kDa counterpart? If so, what are the conditions required to induce this process? Is the processed form still functional? In addition, why are there two forms of acyl-CoA synthetase? How do the two forms compare in terms of their activities and affinities toward the substrates? By answering these questions, we can gain insights into the relationship between any potential regulation mechanisms and the distribution among the two forms of acyl-CoA synthetase.

Although acyl-CoA synthetase from *Escherichia coli* has been the subject of both *in vitro* and *in vivo* studies for over two decades, antibodies against this enzyme are not yet available. As part of the ongoing study of the enzyme, it is essential to have the antibodies to address questions such as: are both forms of the enzyme present *in vivo*? If not, which form of the enzyme is being used under normal physiological conditions? What factors, if any, cause the *in vivo* conversion from one form to the other? All these experiments require a means to take a "snapshot" of acyl-CoA synthetase *in vivo*, since the lysis of the bacteria may be accompanied by non-specific proteolysis of the enzyme and lead to erroneous conclusions. Hence, the best way to determine the *in vivo* state of acyl-CoA synthetase is to boil the whole cells in SDS, followed by SDS-PAGE analysis and probe for the enzyme with antibodies against the protein. Therefore, the raising of antibodies against the protein will facilitate future studies of this enzyme.

2. MATERIALS AND METHODS

2.1 Materials

Escherichia coli strain ML308 was obtained from the American Type Culture Collection. *Escherichia coli* strain JM105 carrying expression plasmid pKK223-3* with the *fadD* insert was obtained from J.H. Yoo from Dr. G.E. Gerber's laboratory, McMaster University, Hamilton, Ontario, Canada. These strains were stored in Luria-Bertani media with 15% glycerol (w/v) at -20°C.

ATP, Brij 58, chloramphenicol, Coenzyme A, EDTA, Freund's Adjuvant (both complete and incomplete), IPTG, α-ketoglutarate, D -lactate, luciferase-luciferin, NAD, PMSF, and Triton X-100 were obtained from Sigma-Aldrich Chemical Company. Equipment and reagents for SDS-PAGE, low range molecular weight markers, and gel filtration chromatography standards were obtained from Bio-rad. The Mono Q column was obtained from Pharmacia. HVLP filters (0.45 μm) were obtained from Millipore. ECL detection kit and Rainbow markers for protein blotting were obtained from Amersham. [9,10-^{3}H]oleic acid and [1-^{14}C]oleic acid were obtained from New England Nuclear. Oleic acid was obtained from Fisher Chemical. Type XAR films were obtained from Eastman Kodak. BCA protein assay kit was obtained from Pierce.

2.2 Cell growth

Escherichia coli ML308 was initially grown in Luria-Bertani contents media at 37°C in a shaking incubator. An aliquot of this culture was subcultured into M9 contents minimal media supplemented with 5 mM oleate, 0.5% Brij 58 (w/v), and 5 μg/ml thiamine (Maloy *et al.*, 1981). The above was allowed to grow at 37°C in a shaking incubator until the culture reached an optical density of 0.6 at 660nm ($\approx 4.8 \times 10^8$ cells/ml). An aliquot of this culture was subcultured again into fresh M9 media with identical supplements and growth conditions. The volume of the aliquot was chosen such that there was at least a 1:100 dilution with the fresh medium. Cells were harvested at a optical density of 0.6 at 660nm by centrifugation at 10,000 ×g at 4°C for 10 minutes. Cells were washed two times in ice-cold M9-minimal media containing 0.5% Brij 58 (w/v).

2.3 Uptake assays

2.3.1 In experiments not involving lactate

An aliquot (500 μl) of washed cell suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) was added to 1 ml of washing buffer containing 150 μg/ml chloramphenicol. The mixture was incubated with shaking at 25°C. After the desired period of starvation, 500 μl of [9,10-^{3}H]oleate in washing buffer containing 100 μg/ml chloramphenicol was added to the incubation mixture to a final oleate concentration of 75 μM and

specific activity of 250 μCi/μmole. At the indicated time point, cells in 250 μl of incubation mixture were isolated by vacuum filtering through a 0.2 μm filter along with 5 ml of ice-cold washing buffer. The filter was washed two times with 5 ml of ice-cold washing buffer. The amount of radioactive oleate taken into the cells was quantified by aqueous scintillation counting of the air dried filters (Mangroo, 1992). Chemiluminesence was controlled by re-counting the samples after they had been set in the scintillation counting machine overnight.

2.3.2 In experiments involving lactate

An aliquot (500 μl) of washed cell suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) was added to 500 μl of washing buffer containing 200 μg/ml chloramphenicol. The mixture was incubated with shaking at 25°C. After the desired period of starvation, 500 μl of washing buffer with 100 μg/ml chloramphenicol and with, or without, 40 μmole of D-lactate was added to the incubation mixture, and the mixture was allowed to incubate for two minutes. Then 500 μl of [9,10-^{3}H]oleate in washing buffer with 100 μg/ml chloramphenicol was added to the incubation mixture to a final oleate concentration of 75 μM and specific activity of 250 μCi/μmole. In the case of double labelling experiments, the radioactive oleate also contained [1-^{14}C]oleate at a specific activity of 25 μCi/μmole. At the indicated time point, cells in 250 μl of incubation mixture were isolated by vacuum filtering through a 0.2

μm filter along with 5 ml of ice-cold washing buffer. The filter was washed two times with 5 ml of ice-cold washing buffer. The amount of radioactive oleate taken into the cells was quantified by aqueous scintillation counting of the air dried filters (Mangroo, 1992).

2.4 Determination of cellular ATP content

2.4.1 Extraction of ATP

An aliquot (500 μl) of washed cell suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) was added to 1 ml of washing buffer containing 150 μg/ml chloramphenicol. The mixture was incubated with shaking at 25°C. At specified times, cellular ATP was isolated by adding 500 μl of the sample to 250 μl of ice-cold 24% perchloric acid and then incubating on ice for 20 minutes. The sample was centrifuged for 5 minutes using a Beckman microcentrifuge at 4°C and 500 μl of the supernatant was added to 250 μl of neutralization buffer containing 2 M KOH and 1 M $KHCO_3$. After 30 minutes on ice, the sample was centrifuged as described above and the supernatant was diluted 10-fold and assayed for ATP (Joshi *et al*, 1989; Bagnara and Finch, 1972).

2.4.2 Quantitation of ATP

An aliquot of the diluted cell extract (50 µl) was added to 100 µl of 135 mM glycylglycine, pH 8.0 containing 37.5 mM $MgCl_2$. After the addition of 100 µl of luciferase-luciferin solution (2 mg/ml), light emission was recorded using a photon counter. A standard curve was established to correlate light emission with known amounts of ATP.

2.5 Determination of cellular coenzyme A level

2.5.1 Extraction of free coenzyme A

An aliquot (500 µl) of washed cell suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) were added to 1 ml of washing buffer containing 150 µg/ml chloramphenicol. The mixture was incubated with shaking at 25°C. At specified times, cellular CoA was isolated by adding 800 µl of the sample to 400 µl of ice-cold 1.2 M perchloric acid with 5mM β-mercaptoethanol and incubating on ice for 20 minutes. The acidic extract was then stored at -20°C overnight. The sample was centrifuged for 5 minutes using a Beckman microcentrifuge at 4°C and 800 µl of the supernatant was added to 400 µl of 0.72 M KOH containing 0.16 M $KHCO_3$. After 30 minutes on ice, the sample was centrifuged as described above and 900 µl of the supernatant was taken for the CoA assay (DeBuysere and Olson, 1983; Corkey, 1988).

2.5.2 Quantitation of free coenzyme A

The fluorometric assay using α-ketoglutarate dehydrogenase to quantitate free CoA was a modification of previously described procedure (Benson *et al*, 1969). An aliquot (850 μl) of neutralized extract was added to 900 μl of assay mix, containing 1.33 mM NAD, 3.5 mM α-ketoglutarate, 110 mM Tris-maleate, pH 7, and 28 mM EDTA in a fluorometric cuvette with constant stirring. The excitation was set at 350 nm and the emission was set at 465 nm. The gradual increase in emission was measured and quantitated by a 0.5 nmole internal standard. Specifically, the linear response range of the amount of free CoA was first established by assaying various known amounts of CoA, and the subsequent addition of the 0.5 nmole CoA internal standard would give a proportionate response.

2.6 Determination of cellular acyl-CoA synthetase level

2.6.1 Release of acyl-CoA synthetase

An aliquot (2.5 ml) of washed cells (2.5 x 10^9 cells/ml) was added to an equal volume of incubation buffer with 150 g/ml of chloramphenicol. The mixture was incubated at 25°C with gyrorotary shaking. At specified times, the mixture was put in ice and cells were immediately lysed by passing through a French Pressure cell. An aliquot (60 μl) of the lysate was assayed for acyl CoA synthetase activity in triplicate.

2.7 Assay for acyl-CoA synthetase

Assay for acyl-CoA synthetase by monitoring [9,10-^{3}H] oleoyl-CoA formation was carried out as described previously (Bar-Tana *et al*, 1971) with modifications. The reaction mixture contained 200mM Tris-Cl, pH 7.5, 16mM $MgCl_2$, 10mM β-mercaptoethanol, 20mM NaF, 0.1% Triton X-100, 10mM ATP, 2.5mM coenzyme A, and 75μM potassium oleate. Enzyme fractions were incubated in the reaction mixture containing all the ingredients except coenzyme A for 2 minutes at 37°C. Coenzyme A was then added to initiate the reaction. Unless otherwise stated, reactions were usually stopped at 3 minutes by removing a 100 μl aliquot from the reaction mixture and adding it to 500 μl of Dole solution (isopropyl alcohol/heptane/1M sulphuric acid, 40:10:1, v/v/v). Water (300 μl), followed by another 300 μl of heptane were also added. The whole mixture was then subjected to vigorous vortexing for 5 seconds. The two phases were allowed to separate. Most of the upper organic phase containing the unreacted radioactive oleic acid was then removed by suction. The lower aqueous phase was then extracted 3 more times with diethyl ether. The radioactivity remaining in the aqueous phase was determined by liquid scintillation counting.

Background values in control reactions which were carried out under identical conditions with all the ingredients except coenzyme A were subtracted from the experimental values.

2.8 Measuring efflux of radioactivity

An aliquot (500 µl) of washed cell suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) was added to 500 µl of washing buffer containing 200 µg/ml chloramphenicol. The mixture was incubated with shaking at 25°C. After the desired period of starvation, 500 µl of washing buffer with 100 µg/ml chloramphenicol and with, or without, 40 µmole of D-lactate was added to the incubation mixture, and the mixture was allowed to incubate for two minutes. Then 500 µl of [9,10-^{3}H]oleate in washing buffer with 100 µg/ml chloramphenicol was added to the incubation mixture to a final oleate concentration of 75 µM and specific activity of 250 µCi/µmole. At the specified time point, a 250 µl aliquot of the incubation mixture was diluted into 750 µl of ice cold washing buffer in Eppendorf tube. Then it was centrifuged for 20 seconds in a Beckman microcentrifuge at maximum speed. 500 µl of the supernatant were added to 500 µl of Dole solution. 300 µl of heptane were added and after vortexing, the upper organic phase was removed. The aqueous phase was extracted 3 times with diethyl ether. The radioactivity remaining in the aqueous phase was determined by liquid scintillation counting. Background level was determined by performing the same extraction in the absence of cells.

2.9 Induced processing of acyl-CoA synthetase

The membrane fraction (100,000×g pellet) was used as the source of the 62 kDa acyl-CoA synthetase. A final concentration of 0.2% Triton X-100, unless specified otherwise, and 100mM Tris-Cl, pH 7.5 was used to induce the processing of the 62 kDa acyl-CoA synthetase to a 45 kDa acyl-CoA synthetase. The reaction was carried out at room temperature for 10 minutes or for the specified period of time. At that time, the reaction was stopped by mixing an equal volume of 2× sample buffer for SDS-PAGE, and boiled immediately.

2.10 Purification of acyl-CoA synthetase

2.10.1 Cell growth

Escherichia coli strain JM105 carrying expression plasmid pKK223-3* with the *fadD* insert was initially grown in Luria-Bertani media with 125μg/ml ampicillin at 37°C with shaking overnight. Then the overnight culture was subcultured into fresh Luria-Bertani media with 125μg/ml ampicillin at a dilution factor of 1:40 and was incubated under the same conditions. The cells were allowed to grow to a density of 4.8×10^8 cells/ml as measured by its optical desity at a wavelength of 660nm. IPTG (1 mM) was added to induce the expression of acyl-CoA synthetase for 75 minutes.

2.10.2 Preparation of 62 kDa acyl-CoA synthetase

Escherichia coli expressing the 62 kDa acyl-CoA synthetase was harvested by centrifugation at 10,000×g for 10 minutes at 4°C. The pellet was washed once with ice cold 50 mM potassium phosphate buffer, pH 7.5. The cells were resuspended in the same buffer and were ready for lysis.

Cells lysis was achieved by passing the resuspended cells through a French Press cell three times. PMSF prepared in isopropyl alcohol and EDTA were both added to a final concentration of 1mM immediately after lysis. Unbroken cells and cell debris were removed by centrifugation at 12,000 ×g for 15 minutes at 4°C.

The cell lysate was then subjected to centrifugation at 100,000 ×g for 1 hour at 4°C to isolate the membrane fraction which was enriched with the 62 kDa acyl-CoA synthetase. The membrane pellet was resuspended with ice cold 50 mM potassium phosphate buffer, pH 7.5. The resuspended membrane fraction was aliquoted, frozen in liquid nitrogen and stored at -80°C as the source of the 62 kDa acyl-CoA synthetase.

2.10.3 Extraction of acyl-CoA synthetase from membrane

The resuspended membrane fraction was incubated in the presence of 0.2% Triton X-100, and 100mM Tris-Cl, pH 7.5 at room temperature for 10 minutes. In the case where the intact 62 kDa acyl-CoA synthetase was desirable, the above incubation was carried out on ice. The incubation mixture

was then subjected to centrifugation at 100,000×g for 1 hour at 4°C. The supernatant was used for the ion exchange chromatography.

2.10.4 Ion exchange chromatography

The detergent extract was applied to a Mono-Q column pre-equilibrated with 100mM Tris-Cl, pH 7.5. After finishing applying the detergent extract to the column, 5 column volumes of the initial buffer were used to wash off excess Triton X-100 from the column. A linear gradient of 100mM Tris-Cl, pH7.5 to 100mM Tris-Cl, pH7.5 with 400mM NaCl was used to elute both the 45 kDa acyl-CoA synthetase and the 62 kDa acyl-CoA synthetase.

2.11 Raising of antibodies against the 45 kDa acyl-CoA synthetase

Purified 45 kDa acyl-CoA synthetase (400μg) was first electrophoresed in a 10% polyacrylamide gel in the presence of SDS and β-mercaptoethanol. The protein band was visualized by staining briefly in Coomassie brilliant blue prepared with distilled water. The band was excised and broken up by passing repeatedly through a needle. This suspension (~1 ml) in 0.85% (w/v) saline was then mixed and emulsified with 1 ml of Freund's complete adjuvant for the initial injection into two specific pathogen free rabbits. Subsequent boosts were carried out as the first injection except that incomplete Freund's Adjuvant was used. These booster injections were done at an interval of at least 6 weeks. Blood samples (5 ml) were taken at around 12 days post injection. The samples

were allowed to clot at 37°C for 30 minutes and let contract at 4°C overnight. The liquid was subjected to centrifugation in a micro-centrifuge for 10 minutes. The serum was stored at -20°C and used as the source of antibodies against the 45 kDa acyl-CoA synthetase.

2.12 Electrophoresis, Immunoblotting and Protein Determination

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described by Laemmli (1970). Separating gels contain 10%, 12.5%, or 15% polyacrylamide, 0.375 M Tris-Cl, pH 8.8, 0.1% SDS, and 1% glycerol. Stacking gels contain 4% polyacrylamide, 0.125 M Tris-Cl, pH 6.8, 0.1% SDS, and 1% glycerol. Proteins were electrophoresed at 200 V in running buffer containing 0.0249 M Tris-glycine, pH 8.3, and 0.1% SDS. Unless otherwise stated, all gels were visualized by staining with Coomassie brilliant blue dye.

Immunoblotting was performed according to the method described in the Bio-rad protein blotting manual. After electrophoresis, the polyacrylamide gel was assembled into a sandwich such that the gel was in contact with a piece of nitrocellulose support. This assembly was immersed into the transfer buffer (Tris-glycine, pH 8.3, with 20% methanol (v/v)). Electrophoretic transfer of protein was carried out at 100 V for 1 hour. The starting temperature was at 4°C, and the transfer was carried out in a 4°C cold room. The nitrocellulose support was prepared according to the method specified in the ECL (Amersham)

manual. Blocking solution was 5% skimmed milk in Tris buffered saline with 0.1% Tween-20 (v/v) (Towbin *et al.*, 1979). Immunodetection was done with polyclonal antibodies raised against the 45 kDa acyl-CoA synthetase in rabbits, as described in Section 2.11 (1:1000 dilution). Goat anti-rabbit IgG antibodies coupled with horse radish peroxidase at a dilution of 1:5000 were used as the secondary antibodies. The presence of primary antibodies was detected by ECL kit and visualized on Kodak XAR film.

Protein determinations were done with the BCA kit using the enhanced protocol and bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

3.1 Effect of starvation on oleate uptake

3.1.1 Oleate uptake within the first minute post starvation

If *Escherichia coli* was starved before measuring its oleate uptake rate, it was observed that the oleate uptake rate during the initial minute of the uptake assay was dramatically lowered in the starved cells compared to the unstarved cells (Figure 3). This was not due to the dying of cells since proline uptake did not change during the course of starvation (Mangroo, 1992). This starvation, which was meant to deplete any carried over fatty acids from the growth media, should therefore increase the observed oleate uptake rate due to the absence of competition against the radioactive oleate by carried over non-radioactive oleate. Since it is an unexpected finding that a bacterium would decrease its nutrient uptake when it is starved, it is then interesting to find out what is the mechanism of this starvation effect on oleate uptake. One possible mechanism is the depletion of ATP during starvation. Others include the depletion of coenzyme A and the decrease in acyl-CoA synthetase level.

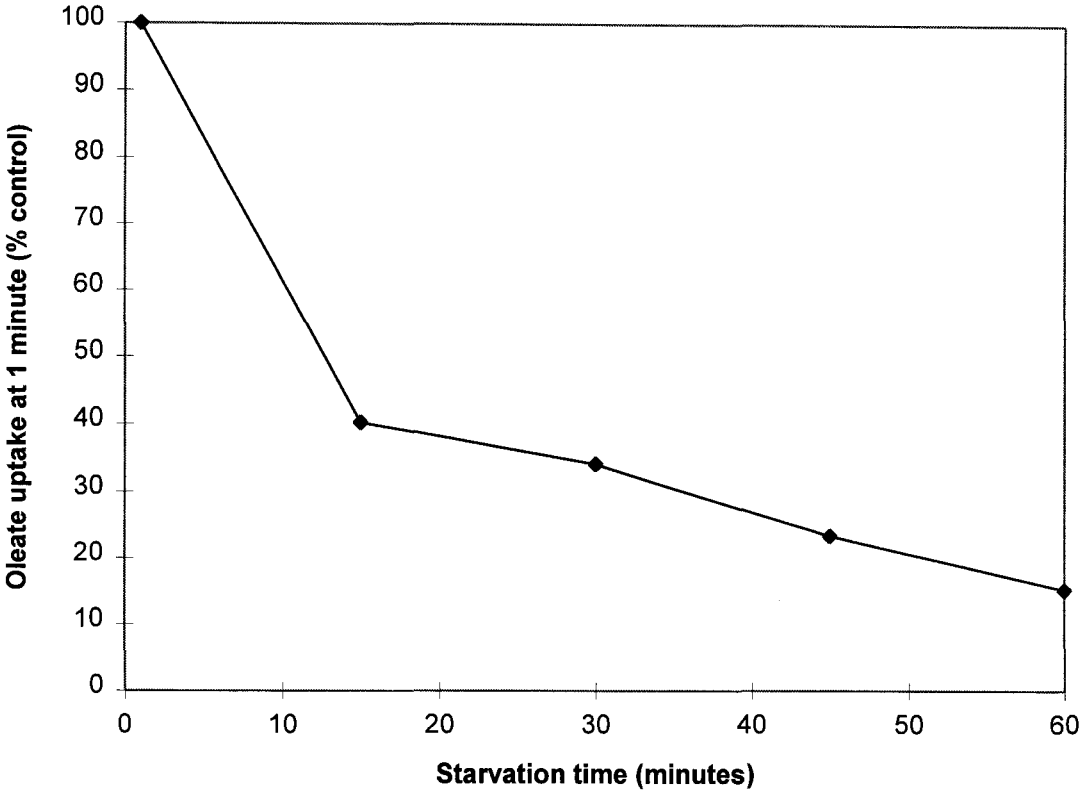

Figure 3. Effect of starvation on oleate uptake in the first minute.

[9,10-^{3}H]Oleate uptake by ML308 was measured after starving the cells for 1(control), 15, 30, 45, and 60 minutes. Specifically, 500 µl of washed cells suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) were added to 1 ml of washing buffer. The starvation mixture was incubated with shaking at 25°C. After the desired period of starvation, 500 µl of [9,10-^{3}H]oleate in washing buffer was added to the incubation mixture to a final oleate concentration of 75 µM and specific activity of 250 µCi/µmole. Chloramphenicol concentration was maintained at 100 µg/ml throughout the experiment. After 1 minute, cells from 250 µl of incubation mixture are isolated by vacuum filtering through a 0.2 µm filter along with 5 ml of ice-cold washing buffer. The filter was washed two times with 5 ml of ice-cold washing buffer. Amount of radioactive oleate taken into the cells was quantitated by aqueous scintillation counting of the air dried filters. Uptake was defined as the amount of radioactivity retained inside cells after incubation with [9,10-^{3}H]oleate for 1 minute.

3.1.2 Cellular ATP level during starvation

Intracellular ATP level was determined during starvation to see if there was a depletion of this substrate. The level of ATP during the course of starvation dropped linearly to approximately 80% of the controlled value (Figure 4). This could not account for the decrease in oleate uptake to 25% of the controlled value (Figure 3). Firstly, the magnitudes of the drop did not correspond to that of initial oleate uptake, and secondly, ATP level dropped in a distinct manner from that of oleate uptake. The ATP level during starvation was consistent with the literature that the depletion of cellular ATP required at least an hour of starvation (Joshi *et al*, 1989).

3.1.3 Cellular coenzyme A level during starvation

Free CoA level during starvation rose to about 200% of its controlled value (Figure 5). This observation that the level of free CoA rose in the absence of an energy source was consistent with that made by others (Jackowski and Rock, 1986; Vallari and Jackowski, 1988). This was somewhat expected, since starvation would mean the depletion of acetyl-CoA which was fed into the tricarboxylic acid cycle for energy production. This also meant that the free CoA

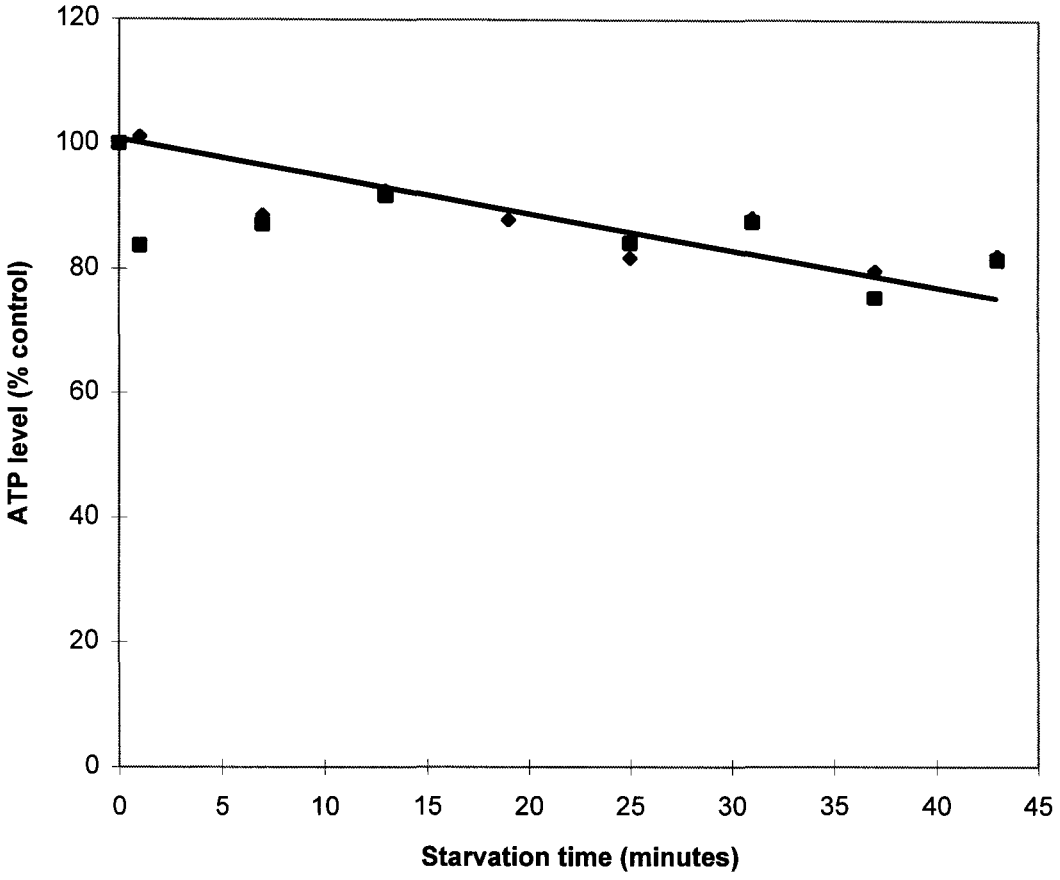

Figure 4. Cellular ATP level during starvation.

Amount of ATP in *Escherichia coli* ML308 during starvation expressed as a percentage of the ATP level before starvation. The two symbols represent a set of data from a duplicate. Specifically, 500 µl of washed cells suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) were added to 1 ml of washing buffer containing 150 µg/ml chloramphenicol. The mixture was incubated with shaking at 25°C. After desired period of starvation, cellular ATP was isolated by adding 500 µl of the sample to 250 µl of ice-cold 24% perchloric acid and incubating on ice for 20 minutes. The sample was centrifuged for 5 minutes using a Beckman microcentrifuge at 4°C and 500 µl of the supernatant was added to 250 µl of neutralization buffer containing 2 M KOH and 1 M $KHCO_3$. After 30 minutes on ice, the sample was centrifuged as described above and the supernatant was diluted 10-fold and assayed for ATP as detailed in Section 2.4.2.

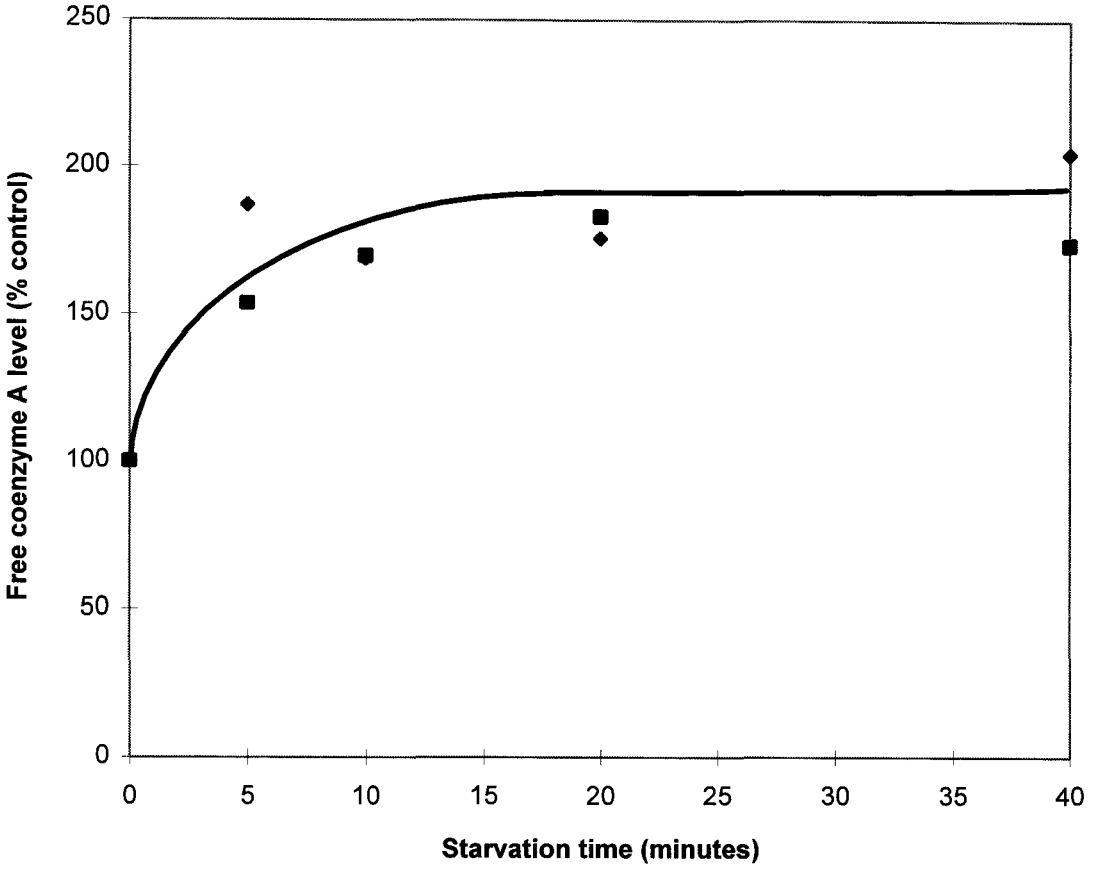

Figure 5. Cellular reduced coenzyme A level during starvation.

Amount of reduced coenzyme A in *Escherichia coli* ML308 during starvation expressed as a percentage of that in unstarved cells. Each symbol represents one data set of a duplicate. Specifically, 500 µl of washed cells suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) were added to 1 ml of washing buffer containing 150 µg/ml chloramphenicol. The starvation mixture was incubated with shaking at 25°C. After desired period of starvation, cellular CoA was isolated by adding 800 µl of the sample to 400 µl of ice-cold 1.2 M perchloric acid with 5mM β-mercaptoethanol and incubating on ice for 20 minutes. The sample was centrifuged for 5 minutes using a Beckman microcentrifuge at 4°C and 800 µl of the supernatant was added to 400 µl of 0.72 M KOH containing 0.16 M $KHCO_3$. After 30 minutes on ice, the sample was centrifuged as described above and 900 µl of the supernatant was taken for the CoA assay as detailed in Section 2.5.2.

produced would not be converted back into acetyl-CoA. Therefore, it is obvious that the availability of free CoA was not a factor causing the observed reduction in oleate uptake during starvation.

3.1.4 Acyl-CoA synthetase level during starvation

In vitro enzyme level was determined. It is clear that the level of acyl-CoA synthetase activity did not drop during starvation (Figure 6). Therefore, the decrease in oleate uptake could not be attributed to a decrease in functional acyl-CoA synthetase level. The apparent increase in activity of the enzyme is expected due to less dilution of the specific activity from carried over non-radioactive oleate of the growth medium. This is why the procedures for fatty acid uptake assay described in the literature always include a starvation step aimed to deplete the carried over non-radioactive fatty acid, which will otherwise decrease the specific activity of the radioactive fatty acid.

3.1.5 Oleate uptake rate between 3 and 9 minutes post starvation

If the oleate uptake rate was measured as the linear oleate uptake rate between 3 and 9 minutes post starvation, then there was no significant effect of starvation on oleate uptake rate (Figure 7). In other words, starvation only affected oleate uptake in the short period of time (1 minute) post starvation. After that, the incubation of the cells in the uptake assay mixture, which contained oleate, was sufficient to reverse the starvation effect. Therefore the

reduction in fatty acid uptake in response to starvation was only temporary. This explains at least partly why the bacteria would survive against the evolutionary pressure to reduce nutrient uptake when it was starved. As to what was causing this temporary effect, the efflux of radioactive probe (discussed below) may hold the answer.

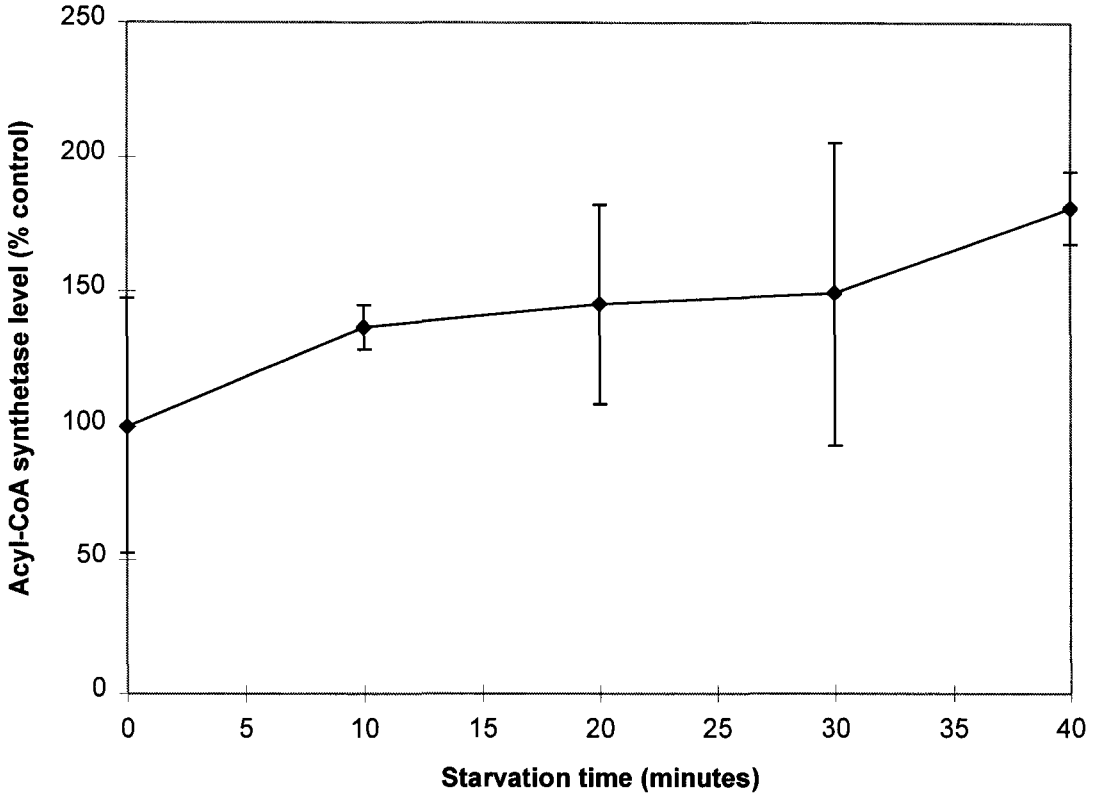

Figure 6. Acyl-CoA synthetase level during starvation of *Escherichia coli* ML308.

Activity of acyl-CoA synthetase during starvation expressed as a percentage of that in unstarved cells. Starvation was carried out as follow, 2.5 ml of washed cells (2.5 x 10^9 cells/ml) was added to an equal volume of incubation buffer with 150 g/ml of chloramphenicol. The mixture was incubated at 25°C with gyrorotary shaking. After the desired period of starvation, the mixture was put in ice and immediately followed by lysis by passing through a French Press cell. An aliquot (60 μl) of the lysate was assayed for acyl CoA synthetase activity. Activity was determined by standard acyl-CoA synthetase activity assays as detailed in Section 2.7. Each data point represents the average value of a triplicate. Error bars are ± S.D..

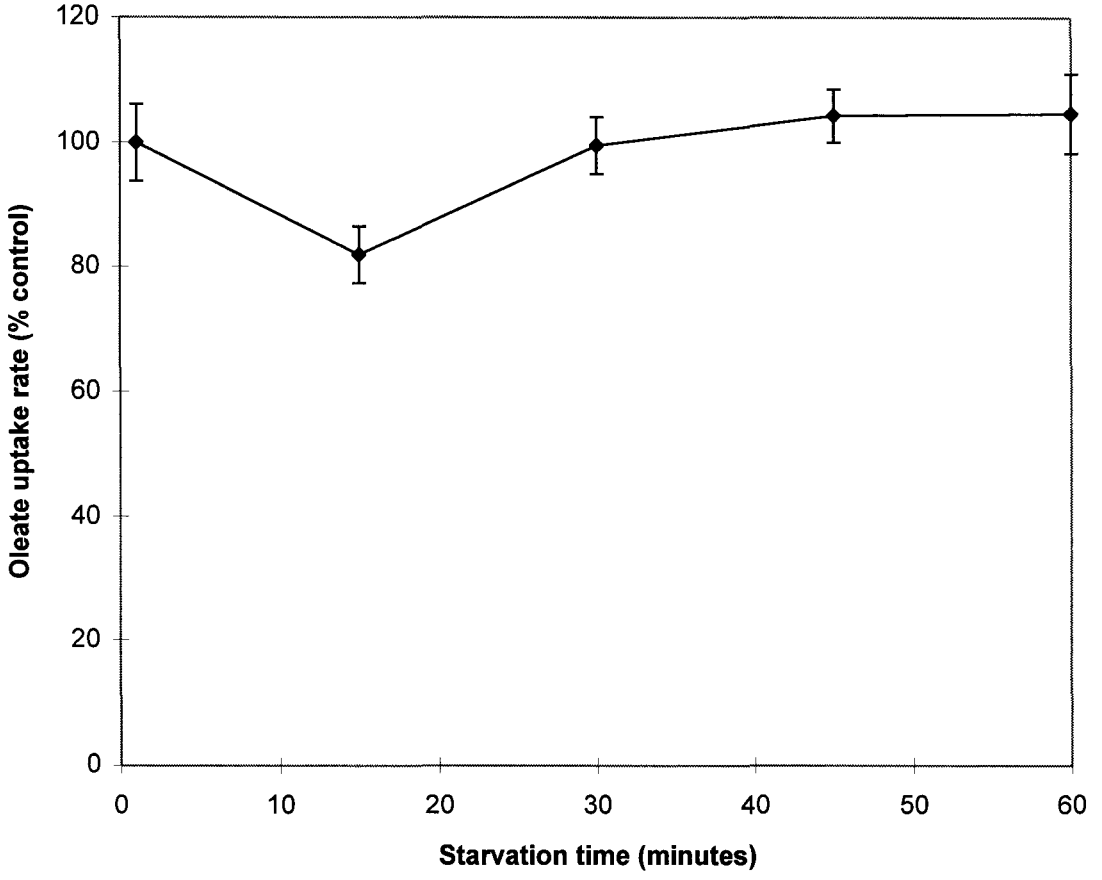

Figure 7. Effect of starvation on oleate uptake rate from 3 to 9 minutes.

[9,10-^{3}H]Oleate uptake by *Escherichia coli* ML308 was measured after starving the cells for 1(control), 15, 30, 45, and 60 minutes. Specifically, 500 µl of washed cells suspension at a density of 2.4×10^9 cells/ml of washing buffer (M9-minimal media with 0.5% Brij 58, w/v) were added to 1 ml of washing buffer. The mixture was incubated with shaking at 25°C. After the desired period of starvation, 500 µl of [9,10-^{3}H]oleate in washing buffer was added to the incubation mixture to a final oleate concentration of 75 µM and specific activity of 250 µCi/µmole. Chloramphenicol concentration was maintained at 100 µg/ml throughout the experiment. At 3, 5, 7, and 9 minutes after the addition of oleate, cells in 250 µl of incubation mixture were isolated by vacuum filtering through a 0.2 µm filter along with 5 ml of ice-cold washing buffer. The filter was washed two times with 5 ml of ice-cold washing buffer. Uptake rate was determined by linear regression on the amount of radioactivity taken up at 3, 5, 7 and 9 minutes after starting incubation with [9,10-^{3}H]oleate.

3.2 Effect of lactate on oleate uptake

3.2.1 Time course of oleate uptake in the presence of lactate

Oleate uptake was clearly higher in the presence of D-lactate. This was true whether *Escherichia coli* ML308 had been starved or not (Figure 8). This suggests D-lactate activates oleate uptake, hence apparently reversing the effect of starvation on oleate uptake at one minute post starvation.

When fatty acid is taken up into *Escherichia coli*, it is first activated by acyl-CoA synthetase to fatty acyl-CoA. From there, it can either enter the β-oxidation pathway to yield acetyl-CoA or it can be incorporated into phospholipids. It was previously shown that in the presence of D-lactate, the amount of both radioactive fatty acyl-CoA and radioactive phospholipids increased upon incubation with radioactive oleate (Mangroo, 1992). In principle, this could be due to i) an increase in the production of fatty acyl-CoA, or ii) a decrease in utilization of fatty acyl-CoA by alternate pathway, i.e., β-oxidation.

Preliminary results had shown that the level of β-oxidation was not significant under the conditions of the uptake assay (Mangroo, personal communication). Since the radioactivity found in the phospholipids must

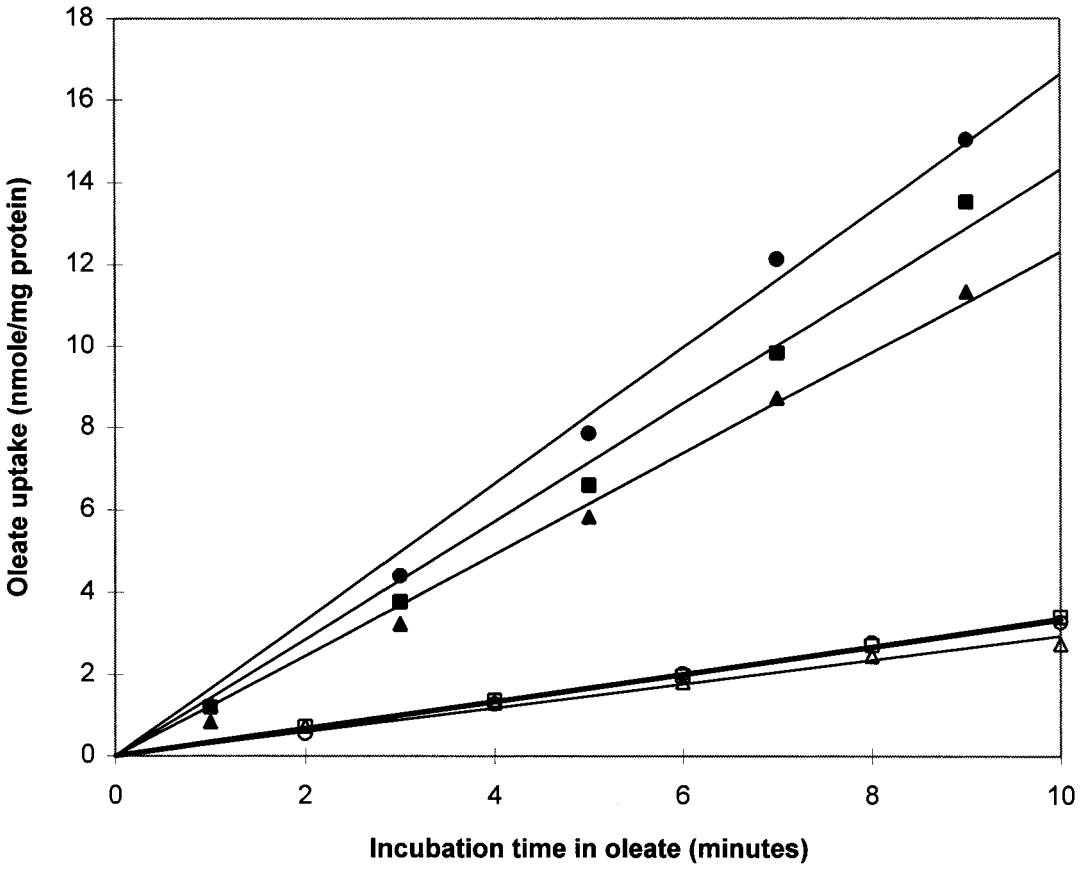

Figure 8. Time courses of oleate uptake in the presence and absence of D-lactate with and without starvation.

[9,10-^{3}H]Oleate uptake by ML308 was measured after starving the cells for 1 (unstarved control) (▲ , △) ;20 (■ , □); and 40 (● , ○) minutes. Then the mixture was incubated for an additional 5 minutes in the presence (▲ , ■ , ●), and absence (△ , □ , ○) of D-lactate as described in Section 2.3.1. [9,10-^{3}H]Oleate was added to the mixture to a final oleate concentration of 75 μM and specific activity of 250 μCi/μmole. At the indicated time, an aliquot of the mixture is vacuum filtered and washed. Uptake was defined as the amount of radioactivity retained on the filter.

originate from fatty acid and hence from fatty acyl-CoA, and the increase in phospholipids level was accompanied by an increase in acyl-CoA level, it was postulated that there had been an elevated level of fatty acyl-CoA production in the presence of lactate (Mangroo, 1992). Because despite the increase in utilization, there was still a higher level of fatty acyl-CoA. This favoured case i) above. However, this bacterium was grown up in oleate as its sole carbon source; it is therefore unreasonable to assume that β-oxidation did not play a significant part in the utilization of fatty acyl-CoA. This leads us to a more thorough investigation of the possibility of case ii) above.

Case ii) states that the increased levels of acyl-CoA could be due to a lower utilization rate of acyl-CoA by another pathway, i.e. β-oxidation, in the presence of D-lactate. This would also explain the fact that radioactive fatty acyl-CoA and phospholipids both increase in the presence of D-lactate. With the inhibition of a step in β-oxidation, the metabolite(s) upstream from the inhibited step will build up. Therefore, there would be a higher level of fatty acyl-CoA thioester, since fatty acyl-CoA thioester is the first metabolite in β-oxidation. This high level of fatty acyl-CoA thioester would be diverted to the other unblocked pathway, namely, to phospholipids synthesis. In fact, the idea that β-oxidation in *Escherichia coli* was regulated has been suggested by others in the literature (Silbert *et al.*, 1972).

The activating effect on fatty acid uptake in *Escherichia coli* has been observed in the presence of other energy sources, including L-lactate, acetate and succinate (Mangroo, 1992). This suggests that the apparent activation could be caused by the presence of an alternate energy source, and hence reducing the need of β-oxidation, since another pathway is also providing the energy requirement for growth.

To relate the above to the apparent activation on oleate uptake by lactate, one must consider the potential of the efflux of radioactive probes from the cells. It is possible that the radioactive [9,10-^{3}H]oleate taken up into the cells is metabolized. The radioactive label can then become associated with another compound, e.g., tritiated water, and can be actively removed from the cells.

According to the above hypothesis, radioactive oleate that is labelled differently may give different results on the uptake assays under identical conditions. In other words, if one observes different results with respect to different radioactive probes, then, there must be efflux of radioactive probe from the cells. This is because different radioactive label would end up in different compounds, e.g. tritium may be transferred to form a reduced FAD or as a (heavy) proton, while the [^{14}C] may end up as $^{14}CO_2$. A double labelling experiment using [9,10-^{3}H]oleate and [1-^{14}C]oleate at the same time can address this question.

3.2.2 Uptake of [9,10-^{3}H] oleate in the presence and absence of lactate

Oleate uptake assays were carried out with both [9,10-^{3}H]oleate and [1-^{14}C]oleate as radioactive probes. Oleate uptake measured with respect to the [9,10-^{3}H] oleate uptake was higher in the presence of lactate than in its absence (Figure 9). As expected, the effect of lactate on oleate uptake was observed as in experiment with [9,10-^{3}H] oleate as the sole radioactive probe.

3.2.3 Uptake of [1-^{14}C] oleate in the presence and absence of lactate

Surprisingly, if oleate uptake was measured by following the radioactivity from the [1-^{14}C] oleate, then there was no effect of starvation on oleate uptake (Figure 10). This favours the notion that there was efflux of metabolized radioactive probes from the cells.

3.2.4 Efflux of radioactivity during uptake assay

To address the question whether there was efflux of radioactivity directly, the extracellular water soluble radioactivity was measured. It was discovered that the amount of water soluble radioactivity found outside cells was higher than that retained inside cells (Figure 12). Since the radioactive oleate added to the reaction mixture would have been removed as oleic acid in the organic

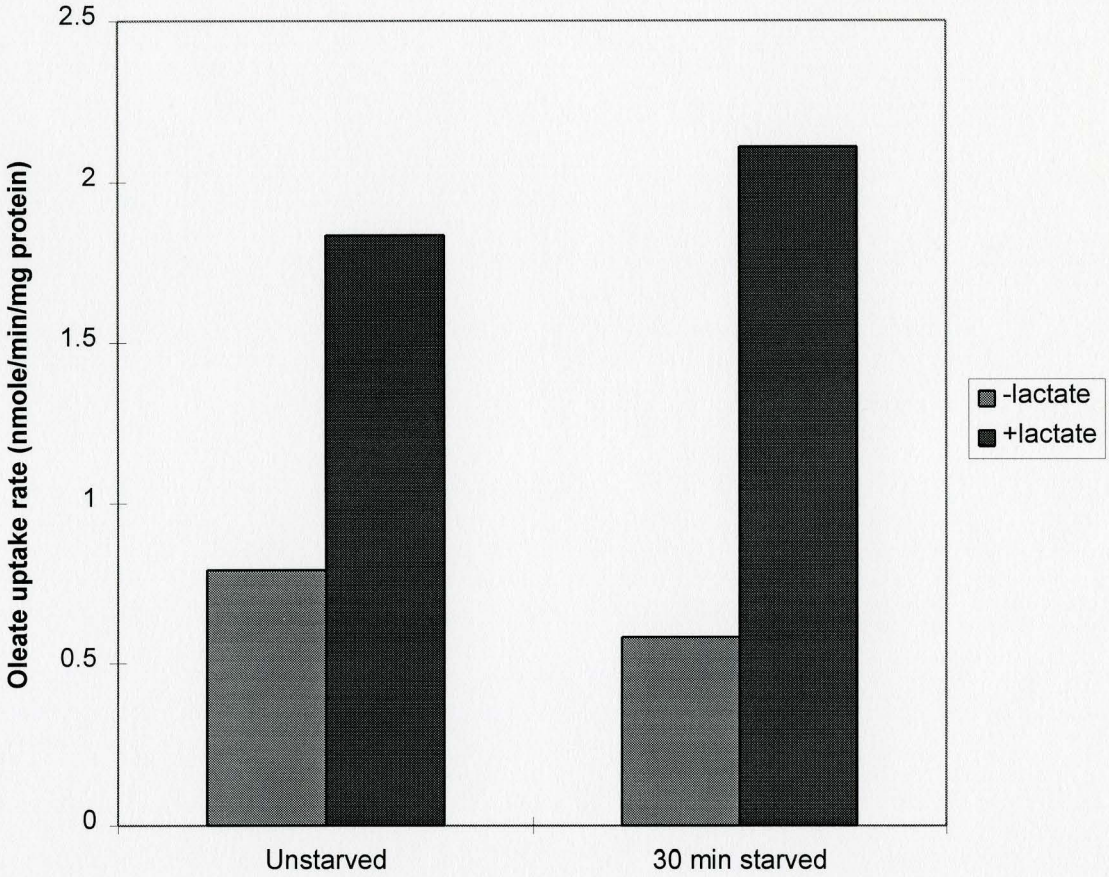

Figure 9. Effect of D-lactate on [9,10-^{3}H] oleate uptake.

Uptake assays were performed as described in Section 2.3.2 with [9,10-^{3}H] oleate and [1-^{14}C] oleate as radioactive probes. Rates were obtained from linear regression of the amount of oleate uptake at 1,2,3, and 5 minutes, with respect to ^{3}H counts. The effect of D-lactate on both starved and unstarved *Escherichia coli* ML308 was investigated.

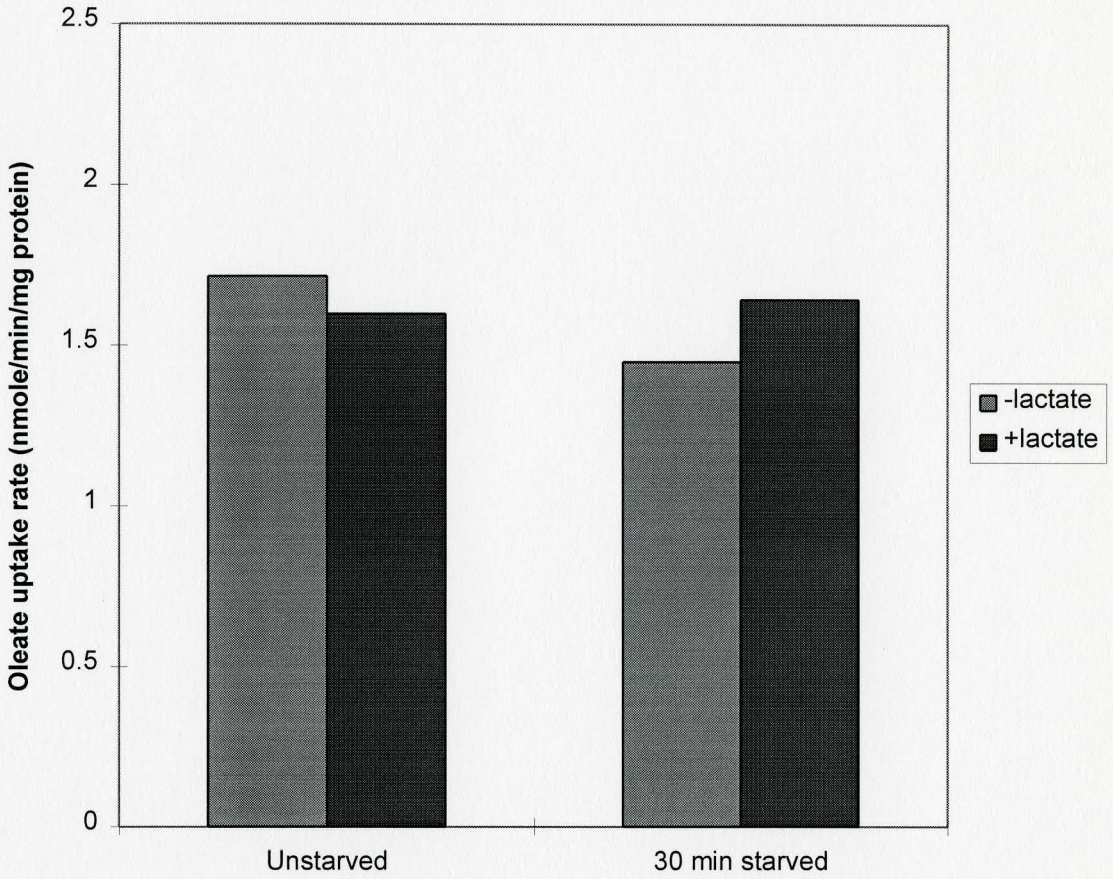

Figure 10. Effect of D-lactate on [1-^{14}C] oleate uptake.

Uptake assays were performed as described in section 2.3.2 with [9,10-^{3}H] oleate and [1-^{14}C] oleate as radioactive probes. Rates were obtained from linear regression of the amount of oleate uptake at 1,2,3, and 5 minutes, with respect to ^{14}C counts. The effect of D-lactate on both starved and unstarved *Escherichia coli* ML308 was investigated.

phase during extractions under acidic conditions, the radioactivity that remained in the aqueous phase during extractions with diethyl ether must be some metabolized product of the radioactive oleate used in the uptake assay. Of course, the amount of radioactive impurities and the residual oleic acid that stayed in the aqueous phase was subtracted from the experimental values (Section 2.8). The oleate, once inside cells, was metabolized and the radioactive labels ended up in forms other than oleate. It is possible that the tritium label on the radioactive oleate was being abstracted by 3-hydroxyacyl-CoA dehydrogenase (Figure 11), and was actively removed from cells in the process of generating the proton gradient across the inner membrane.

3.2.5 Effect of lactate on total oleate uptake

As mentioned earlier, lactate apparently activated oleate uptake as determined by the standard filter assay. However, lactate also had an inhibitory effect on efflux of radioactive probes (Figures 12 and 13). If total oleate uptake was defined as the sum of the radioactivities which remained inside cells and those that were metabolized and effluxed, then lactate had no effect on total oleate uptake (Figures 12 and 13). Therefore, the effect of lactate was not on the uptake of oleate, but rather it was on the efflux of radioactive probes.

Since the majority of radioactivity was metabolized and effluxed to the external medium during the uptake assay, and the efflux of radioactive probes was affected by the metabolic state of the bacterium, the radioactivity

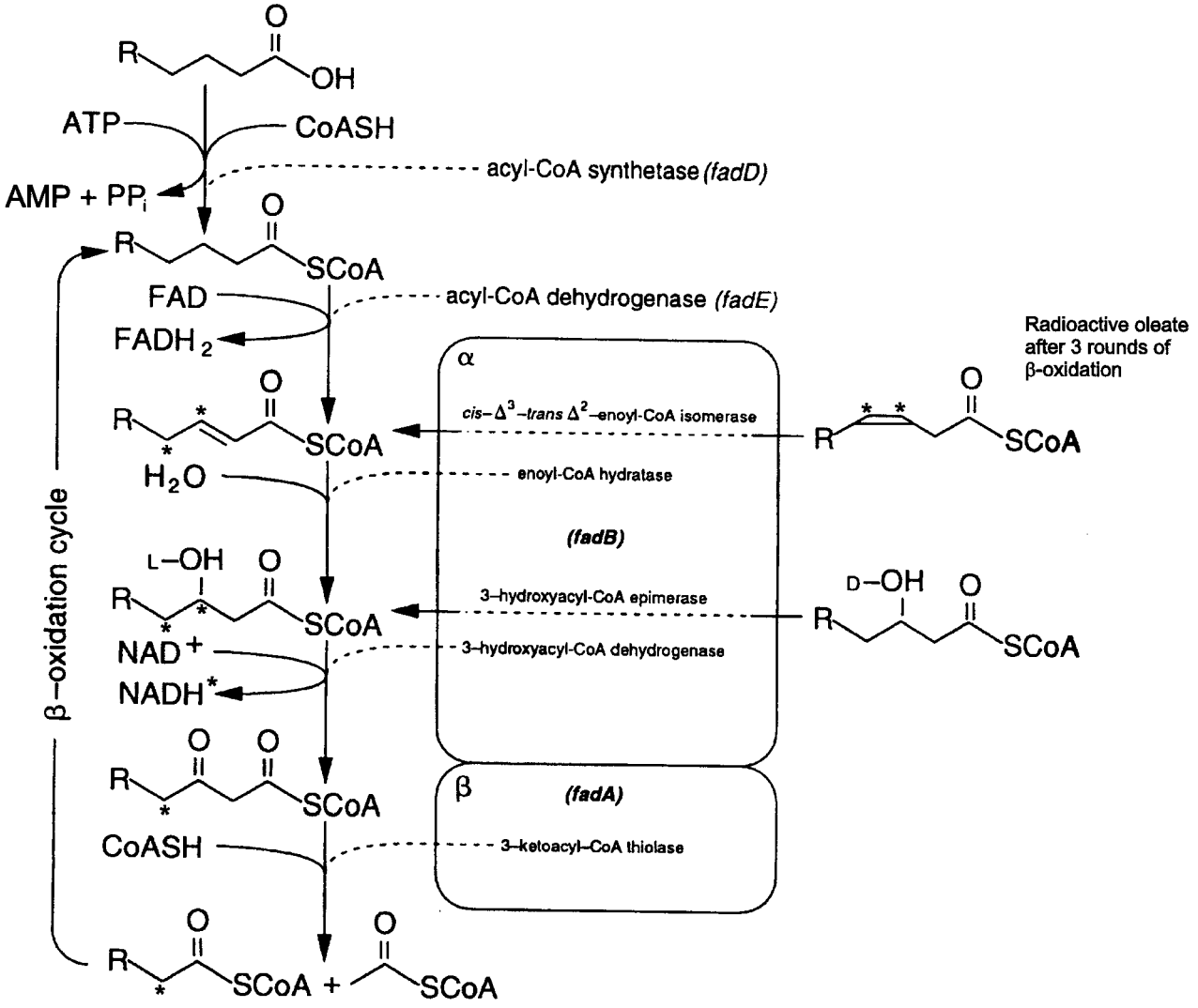

Figure 11. A scheme to denote a possible fate of the tritium label in [9-10]-labelled oleate. Tritium is attached to carbon labelled with an (*). The tritium attached to carbon 9 may be abstracted by 3-hydroxyacyl-CoA dehydrogenase as shown.

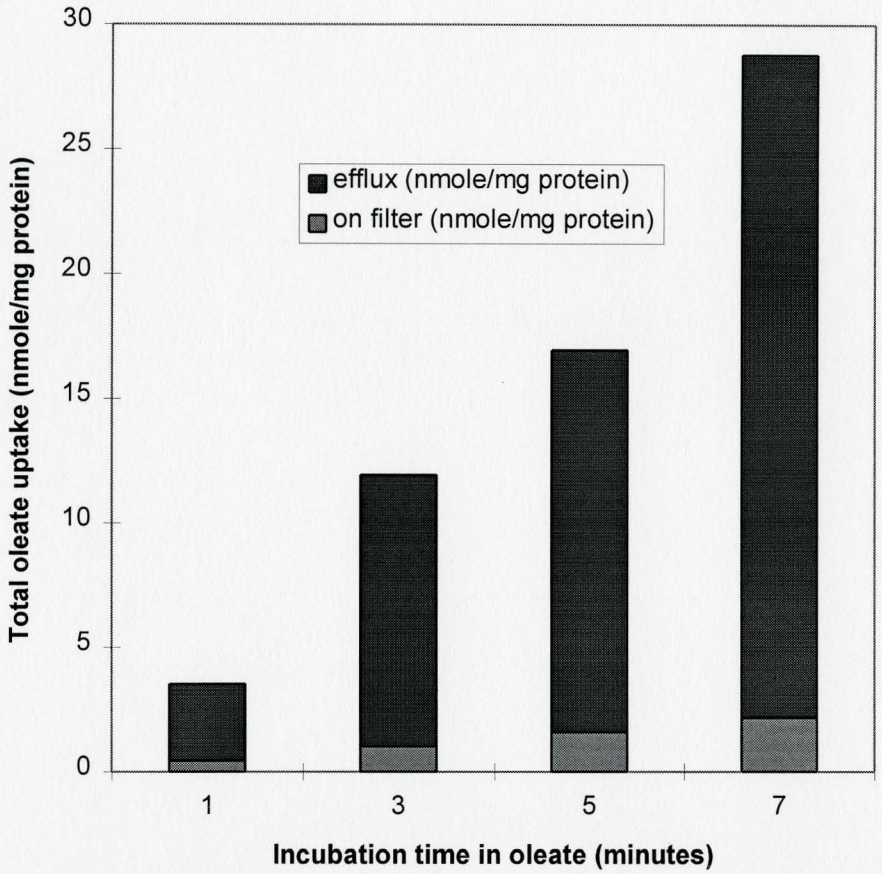

Figure 12. Total oleate uptake and efflux of radioactive probes in the absence of D-lactate.

Total oleate uptake is defined as the amount of radioactivity trapped on the filter plus the water soluble radioactivity found outside cells (efflux). Radioactivity retained inside cells was determined by filter assay as described in Section 2.3.2 without lactate. Efflux in cells, that received identical treatment used in filter assay, was measured by diluting 250 µl of the incubation mixture into 750 µl of ice cold washing buffer in Eppendorf tube. Then it was centrifuged for 20 seconds in a Beckman microcentrifuge at 4°C. 500 µl of the supernatant were added to 500 µl of dole solution. 300 µl of heptane were added and after vortexing, the upper organic phase was removed. The aqueous phase was extracted 3 times with diethyl ether. The radioactivity remaining in the aqueous phase (efflux) were determined by liquid scintillation counting.

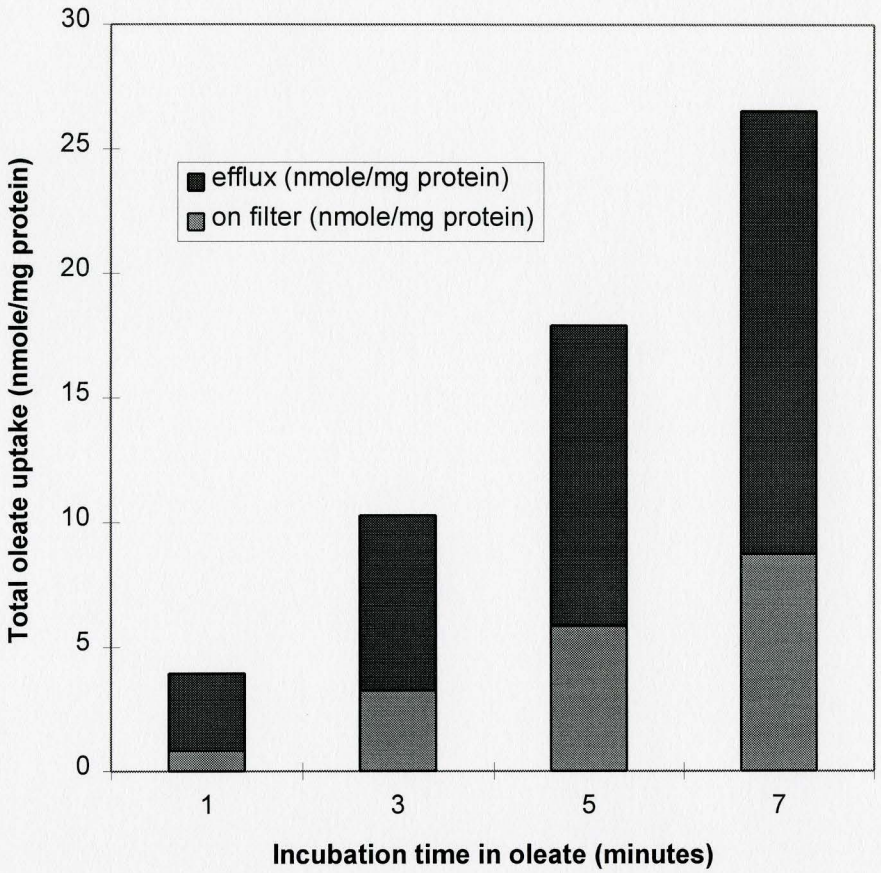

Figure 13. Total oleate uptake and efflux of radioactive probes in the presence of D-lactate.

Total oleate uptake is defined as the amount of radioactivity trapped on the filter plus the water soluble radioactivity found outside cells (efflux). Radioactivity retained inside cells was determined by filter assay as described in Section 2.3.2 with lactate. Efflux in cells, that received identical treatment used in filter assay, was measured by diluting 250 µl of the incubation mixture into 750 µl of ice cold washing buffer in Eppendorf tube. Then it was centrifuged for 20 seconds in a Beckman microcentrifuge at 4°C. 500 µl of the supernatant were added to 500 µl of dole solution. 300 µl of heptane were added and after vortexing, the upper organic phase was removed. The aqueous phase was extracted 3 times with diethyl ether. The radioactivity remaining in the aqueous phase (efflux) were determined by liquid scintillation counting.

retained on the filters did not fully represent the total level of fatty acid uptake. However, this type of fatty acid uptake assay using filters has been used routinely in this research field without taking into consideration the efflux of the radioactivity (Black, 1988; Mangroo, 1992; Kumar and Black, 1993; Azizan and Black, 1994; Black and Zhang, 1995). This negligence may cause erroneous interpretation of experimental results. For example, failure to show an activating effect of lactate on fatty acid uptake has been used in the literature as a characteristic of a defect on fatty acid transport in a *tsp*$^-$ mutant (Azizan and Black, 1994). It is unclear at this time what caused the *tsp*$^-$ mutant's failure to respond to lactate, or what is the site of interaction of the tsp with fatty acid metabolism. Based on our results, it would seem likely that the failure to respond to lactate in the *tsp*$^-$ mutant is the result of the absence of an inhibitory effect on the efflux of radioactive probes derived from [9,10-^{3}H]oleate.

3.3 Two forms of acyl-CoA synthetase

Acyl-CoA synthetase has previously been purified as a 45 kDa enzyme (Kameda and Nunn, 1981; Kameda *et al.*, 1985). However, the gene sequence predicted a 62 kDa protein (Black *et al.*, 1992). In fact, Black *et al.* had shown that the cloned, overexpressed acyl-CoA synthetase was expressed as a 62 kDa protein. Based on the above, and together with gene sequence analysis, Black *et al.* suggested that there was no evidence for processing of acyl-CoA synthetase (Black *et al.*, 1992). In other words, there had been two forms of acyl-CoA synthetase from *Escherichia coli* that were described in the literature, and the functional significance of the existence of the enzyme in two forms is unclear. Are these two forms of the enzyme related? Can the 62 kDa acyl-CoA synthetase be converted to its 45 kDa counterpart? If so, what are the conditions that are required to induce this process? Is the processed form still functional?

3.4 Processing of acyl-CoA synthetase

The overexpression system used in the present study produced a 62 kDa acyl-CoA synthetase. In this study the processing of the enzyme was investigated. In the presence of Triton X-100, this protein could be converted

into a 45 kDa acyl-CoA synthetase *in vitro* which was still active. Some properties of this *in vitro* process will be discussed below.

3.4.1 Time course of processing

In the presence of the detergent Triton X-100, the membrane bound 62 kDa acyl-CoA synthetase was processed in a time dependent manner into its 45 kDa counterpart (Figure 14). A ~17 kDa protein appeared concomitantly with the 45 kDa acyl-CoA synthetase. This suggests that the 45 kDa protein was derived from the 62 kDa acyl-CoA synthetase, and the 17 kDa protein was the fragment being cleaved off from the 62 kDa acyl-CoA synthetase.

3.4.2 Effect of Triton X-100 concentration on processing and extraction of acyl-CoA synthetase from cytoplasmic membrane

The processing of the 62 kDa acyl-CoA synthetase into the 45 kDa acyl-CoA synthetase was induced by Triton X-100. In the absence of the detergent, little processing was observed even after one hour of incubation at room temperature (Figure 14, control). Although no 62 kDa acyl-CoA synthetase was seen to dissociate from the membrane by washing the membrane fraction in the absence of detergent (Figure 15, control), the induction of processing was a separate process from the extraction of acyl-CoA synthetase from the membrane. At 0.1% Triton X-100, essentially all acyl-CoA synthetase was extracted from the membrane, yet there was still some 62 kDa acyl-CoA

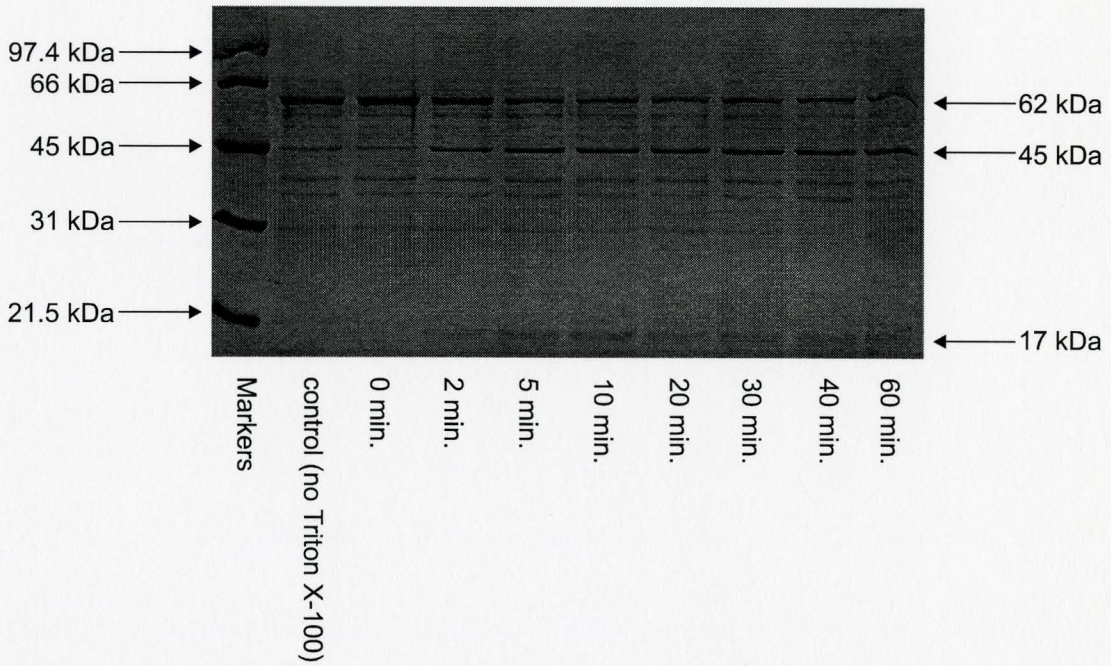

Figure 14. Time course of Triton X-100 induced processing of acyl-CoA synthetase. Membrane fraction were prepared as described in Section 2.10.2. Incubation mixture (1 ml, containing 0.2% Triton X-100 (w/v), 100 mM Tris-Cl, pH7.5, and 307 mg of protein from membrane fraction) was incubated at room temperature for the indicated period of time. The control reaction was incubated for one hour at room temperature in the absence of detergent. Samples were analyzed by SDS-PAGE in a 12% gel as detailed in Section 2.12. Each lane contained 3 mg of protein.

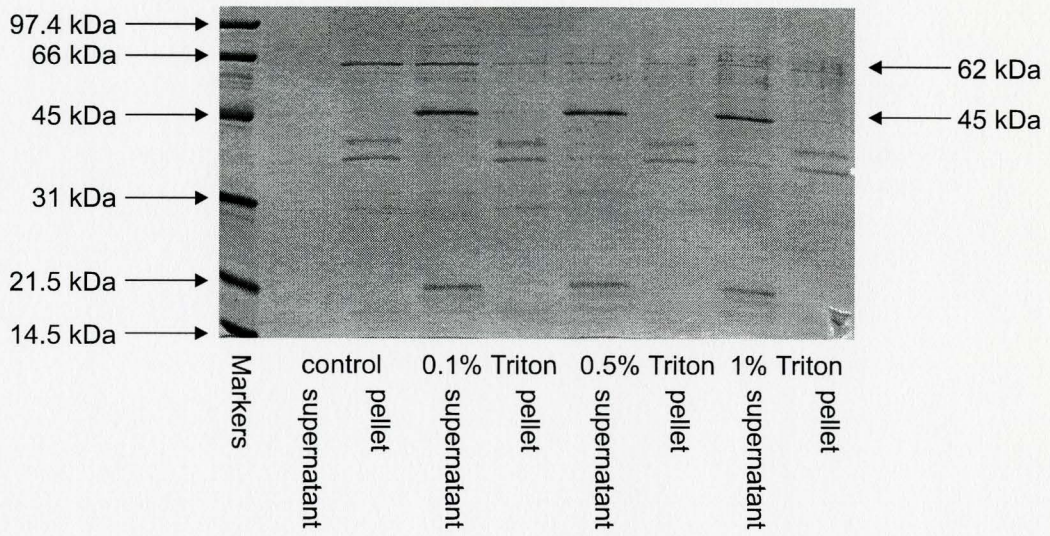

Figure 15. Effect of Triton X-100 concentration on extraction and processing. Resuspended membrane (Section 2.10.2) was used as source of the 62 kDa acyl-CoA synthetase. Incubation was carried at room temperature for 10 minutes in the presence of the specified concentration of Triton X-100. One hour of centrifugation at 100,000´g at 4°C isolated the residual membrane (pellet) from the soluble fraction (supernatant). Control contained no Triton X-100. Samples were analyzed by SDS-PAGE in a 12% gel as detailed in Section 2.12.

synthetase remaining unprocessed (Figure 15). This indicates that it is possible to dissociate the 62 kDa acyl-CoA synthetase from the membrane without processing the enzyme into the 45 kDa form. At higher Triton X-100, the only form of acyl-CoA synthetase observed was the 45 kDa (Figure 15).

3.4.3 Effect of oleate on processing

The processing of the 62 kDa acyl-CoA synthetase induced by Triton X-100 was inhibited by oleate, which is a natural substrate of the enzyme (Figure 16). This inhibitory effect of oleate on processing was dependent on the concentration of oleate (Figure 17). It is possible that Triton X-100 induces processing by removing free oleate from the solution by forming mixed micelles with oleate. Since the majority of oleate would partition into the detergent micelles, the free concentration of oleate would be drastically lowered in the presence of Triton X-100. On the other hand, the addition of more and more exogenous oleate would eventually restore the original free oleate concentration. Although exogenous oleate had to be added to a final concentration of around 300 μM to 400 μM before its inhibitory effect could be observed, the free oleate concentration in the incubation mixture was much lower than the 300 μM level. This is because most of the added oleate would partition into the Triton X-100 micelle, thus maintaining a low free oleate concentration.

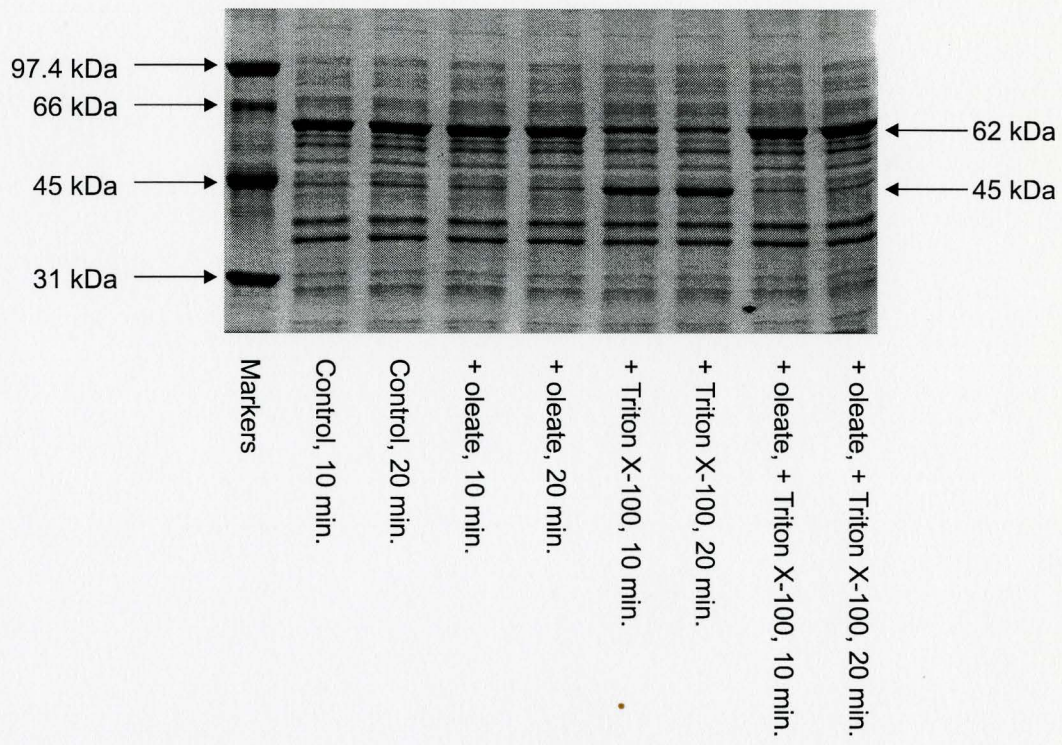

Figure 16. Inhibitory effect of oleate on processing.
All incubations were carried out at room temperature for the indicated period of time. The + Triton X-100 contained 0.2% (w/v) of the detergent. The + oleate contained 3.75 mM of oleate. Membrane fraction (Section 2.10.2) was used as a source of the 62 kDa acyl-CoA synthetase. Experiment was carried out as described in Section 2.9. Control contained no Triton X-100 or oleate. Samples were analyzed by SDS-PAGE in a 12% gel as detailed in Section 2.12.

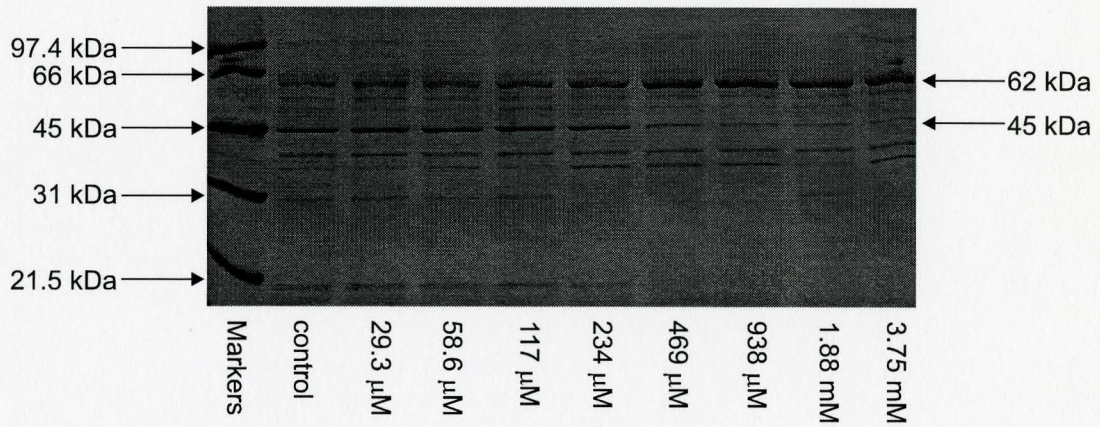

Figure 17. Dependence of processing on oleate concentration.
Experiments were done as detailed in Section 2.9. Membrane fraction (Section 2.10.2) was used as a source of the 62 kDa acyl-CoA synthetase. Triton X-100 (0.2%) were used in all reactions. The indicated concentrations of oleate were included in the incubation mixture. All reactions were carried out at room temperature for 20 minutes. Control contained no oleate. Samples were analyzed by SDS-PAGE in a 12% gel as detailed in Section 2.12.

3.4.4 Relationship between the two forms of acyl-CoA synthetase

Figure 14 suggests the 45 kDa acyl-CoA synthetase was derived from the 62 kDa acyl-CoA synthetase, because the appearance of the 45 kDa band corresponded to the disappearance of the 62 kDa band. To gather further evidence that the 45 kDa acyl-CoA synthetase is derived from the 62 kDa form, antibodies raised against the 45 kDa acyl-CoA synthetase were used. It was shown that the antibodies recognized the 62 kDa acyl-CoA synthetase (Figure 18). Although the antibodies showed cross-reactivity to some other proteins as well, the finding that the antibodies against the 45 kDa acyl-CoA synthetase recognized the 62 kDa acyl-CoA synthetase is consistent with the notion that the 45 kDa acyl-CoA synthetase was derived from the 62 kDa form.

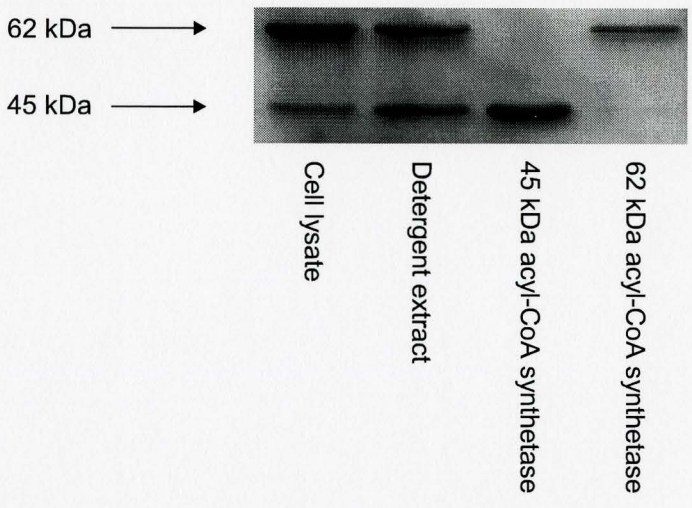

Figure 18. Immunological relationship between the two forms of acyl-CoA synthetase. Samples were prepared as described in Sections 2.10.2-4. Western blot was performed using 10% SDS polyacrylamide gel and nitrocellulose, and detected with polyclonal antibodies raised against the 45 kDa acyl-CoA synthetase, as described in Section 2.11 (1:1000 dilution). The presence of primary antibodies was detected by ECL kit, as described in Section 2.12., and visualized on Kodak XAR film.

3.5 Purification of acyl-CoA synthetase

3.5.1 Membrane associated form of acyl-CoA synthetase

It is interesting to note that almost all of the acyl-CoA synthetase that is contained in the membrane fraction is that of the 62 kDa size. This can be seen on the silver stained polyacrylamide gel (Figure 19 and Figure 20). In the membrane fraction lane, there was no significant band at around 45 kDa. At the same time, the 62 kDa band was the prominent band. This suggests that the 62 kDa form of acyl-CoA synthetase is membrane associated.

The practical implication of this enzyme being membrane associated is the ease of purification. The membrane fraction contained the 62 kDa acyl-CoA synthetase as the major protein. Therefore, by removing the cytosolic fraction, there was an automatic 3 fold purification (Figure 19, Table 1). Also, since the cytosolic fraction was discarded early in the purification procedure, the waste of the relatively abundant starting materials could be justified. In addition, scaling up the process to this stage did not impose any pressure in terms of capacity to any step along the purification scheme thus far.

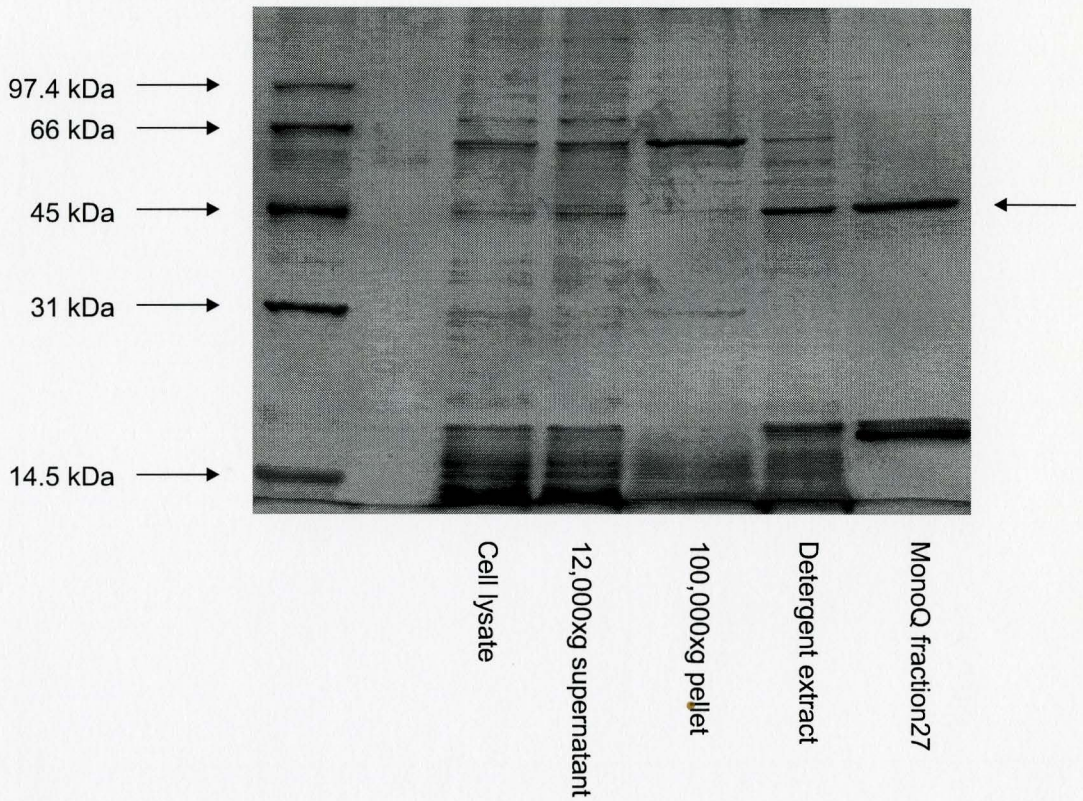

Figure 19. SDS-PAGE analysis on the purification of the 45 kDa acyl-CoA synthetase. Samples from each step of the purification procedure were run on a 15% polyacrylamide gel in the presence of SDS and β-mercaptoethanol. Each lane contained 0.89 μg of protein. Protein bands were visualized by silver staining.

Procedure	Volume (ml)	Total activity (U)	Specific activity (U/mg protein)	Purification (-fold)	Yield (%)	Total protein (mg)
Cell lysate	24	22100	104	1	100	210
Membrane fraction	10	10800	352	3.4	49	31
Detergent extract	100	5320	231	2.2	24	23
Mono Q	11	636	648	6.3	2.9	0.98

Table 1. Summary of purification of the 45 kDa acyl-CoA synthetase.

Acyl-CoA synthetase activities were determined by standard assay using [9,10-^{3}H]oleate. One unit of activity represents the formation of 1 nmole of oleoyl-CoA per minute per milligram of protein. Membrane fraction refers to 100,000×g pellet; detergent extract refers to the 100,000×g supernatant of membrane fraction after incubation in 0.2% Triton X-100; and Mono Q refers to fraction #27 from the anion exchange column that contains the 45 kDa acyl-CoA synthetase with the least contaminating proteins.

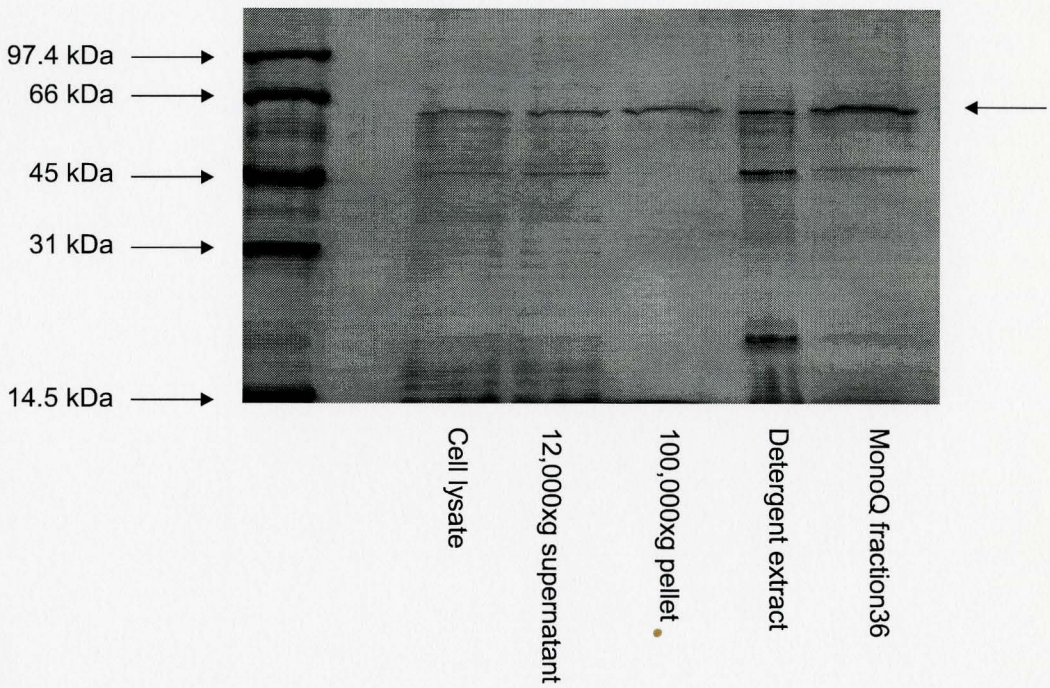

Figure 20. SDS-PAGE analysis on the purification of the 62 kDa acyl-CoA synthetase. Samples from each step of the purification procedure were run on a 15% polyacrylamide gel in the presence of SDS and β-mercaptoethanol. Each lane contained 0.15 μg of protein. Protein bands were visualized by silver staining.

Procedure	Volume (ml)	Total activity (U)	Specific activity (U/mg protein)	Purification (-fold)	Yield (%)	Total protein (mg)
Cell lysate	24	22100	104	1.0	100	210
Membrane fraction	10	10800	352	3.4	49	30
Detergent extract	100	8130	357	3.4	37	23
Mono Q	11	182	1130	11	0.80	0.16

Table 2. Summary of purification of the 62 kDa acyl-CoA synthetase.

Acyl-CoA synthetase activities were determined by standard assay using [9,10-^{3}H]oleate. One unit of activity represents the formation of 1 nmole of oleoyl-CoA per minute per milligram of protein. Membrane fraction refers to 100,000×g pellet; detergent extract refers to the 100,000×g supernatant of membrane fraction after incubation in 0.2% Triton X-100; and Mono Q refers to fraction #36 from the anion exchange column that contains the 62 kDa acyl-CoA synthetase with the least contaminating proteins.

3.5.2 Detergent extraction of membrane associated acyl-CoA synthetase

With 0.2% Triton X-100 (w/v), essentially all membrane bound acyl-CoA synthetase can be extracted from the membrane. To obtain the 45 kDa processed form of acyl-CoA synthetase, a 10 minute incubation in Triton X-100 at room temperature, followed by an one hour centrifugation at 100,000×g at 4°C would result in almost all acyl-CoA synthetase in the 45 kDa form (Figure 19). However, if the incubation was carried out on ice, then about half of the extracted acyl-CoA synthetase remained as the 62 kDa form (Figure 20).

A significant loss of acyl-CoA synthetase activity was observed during this step. It had been reported previously that *Escherichia coli* inner membrane vesicles would activate acyl-CoA synthetase activity by two fold (Mangroo and Gerber, 1993). Therefore, the removal of membrane during the process may contribute to this significant loss of activity. The difference in loss in activity between the room temperature incubation and the ice cold incubation (Table 1 and Table 2) during this step was probably due to the difference in activity level of the two forms of acyl-CoA synthetase towards oleate (will be discussed in Section 3.6). In fact incomplete extraction from membrane should not be a factor, since there was no significant amount of acyl-CoA synthetase associated with the membrane pellet after detergent extraction (Figure 15). Also, although loss in acyl-CoA synthetase activity amounted up to 50%, only 25% loss of protein was recorded during this step (Table 1).

3.5.3 Purification by ion-exchange chromatography

The only chromatographic step in the purification scheme involved the use of the anion exchange column Mono Q. Triton X-100 introduced to the sample earlier was removed during this step. Triton X-100 is a non-ionic detergent and would not bind to the positively charged quaternary amine groups of Mono Q. Therefore, washing the column with 5 bed volumes of starting buffer helped to remove the detergent. Fraction 27 was used as a source of 45 kDa acyl-CoA synthetase (Figure 19).

Attempts to purify the 45 kDa band from the lower band at around 17 kDa have not yet been successful. This lower band co-isolated with the 45 kDa acyl-CoA synthetase in gel filtration chromatography using Superdex-75 column. The complex migrated at around the combined size of 62 kDa (Data not shown). It is interesting to note that if the whole fraction 27 was reapplied to Mono Q column after dilution, with identical elution conditions, two peaks appeared in the elution profile. The first peak corresponded to the elution volume of fraction 27, as expected. The second peak, however, appeared at a much later elution volume where usually the majority of the 62 kDa acyl-CoA synthetase was found (Data not shown). It is possible that the 17 kDa fragment was still associated with the 45 kDa acyl-CoA synthetase as a heterodimer, and sometimes behaved as the 62 kDa acyl-CoA synthetase during ion-exchange chromatography.

In establishing the purity of fraction 27, SDS-PAGE analysis using 15% gel was employed. In previously published purification scheme, a 8% cross linking gel was used (Kameda and Nunn, 1981). Under the latter condition, a 17 kDa band would have migrated off the gel.

3.6 Kinetics analysis of acyl-CoA synthetase

The standard assay conditions have 0.1% Triton X-100 (w/v) in the reaction mixture (Bar-Tana *et al.*, 1971; Kameda and Nunn, 1981; Kameda *et al.*, 1985). On the other hand, oleate, as a salt of a fatty acid or a detergent, will inevitably partition into the Triton X-100 micelles to form mixed micelles. The exact behaviour of acyl-CoA synthetase catalysis in terms of enzyme localization is not known, although there has been evidence that membranous surface is required for optimal activity of this enzyme (Mangroo, 1992; Mangroo and Gerber, 1993). Therefore interfacial catalysis should be considered in the analysis. Investigations on phospholipase A_2, which uses phospholipids as substrates, have provided a model system in the studies of interfacial catalysis. However, to complicate things further, oleate, unlike phospholipids, has a relatively high critical micelle concentration. The critical micelle concentration for phospholipids is below 0.1 nM, this low monomer concentration prevents

intervesicle exchange of phospholipids (Ramirez and Jain, 1991). In the case of oleate, which has a critical micelle concentration in the range of mM, there should be a significant intermicelle exchange rate. Therefore, the kinetics parameters determined in the present study are aimed for the comparison between the parameters of the two forms of acyl-CoA synthetase under standard assay conditions, and should be viewed with such limitation in mind.

The K_M and V_{max} for the 45 kDa acyl-CoA synthetase were determined to be 85 ± 18 μM and 1550 ± 165 nmole/min/mg protein respectively (Figure 21). Whereas the K_M and V_{max} for the 62 kDa acyl-CoA synthetase were determined to be 38 ± 12 μM and 633 ± 79 nmole/min/mg protein respectively (Figure 22). This suggests that the 62 kDa enzyme has higher affinity towards oleate than its 45 kDa counterpart, at least under standard assay conditions. Interestingly, the ratio of V_{max}/K_M remained relatively constant between the two forms of the enzyme.

3.7 Effect of Triton X-100 on the activity of acyl-CoA synthetase

It has been previously reported that partially purified acyl-CoA synthetase can be activated by inclusion of 0.1% Triton X-100 in the reaction mixture (Mangroo and Gerber, 1993). However, it was not known which form of the acyl-CoA synthetase was being affected.

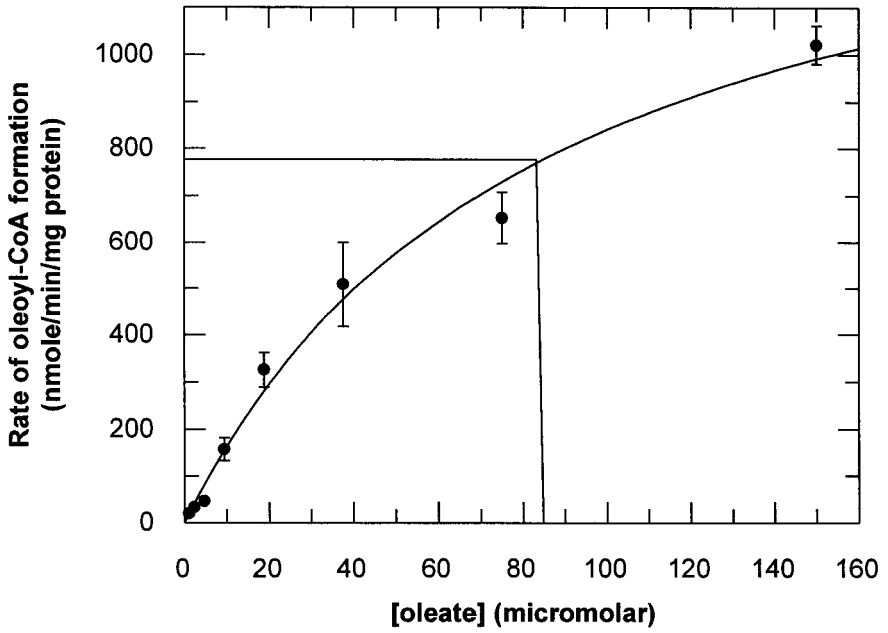

Figure 21. A plot of the initial rate versus the substrate concentration for the 45 kDa acyl-CoA synthetase.

K_M and V_{max} were determined to be 85 ± 18 μM and 1550 ± 165 nmole/min/mg protein respectively by fitting data directly into the Michaelis-Menten equation. Each points represented the average of a triplicate, and error bars were ± S.D.. The reaction mixture contained 200mM Tris-Cl, pH 7.5, 16mM $MgCl_2$, 10mM β-mercaptoethanol, 20mM NaF, 0.1% Triton X-100, 10mM ATP, 2.5mM coenzyme A, and 75μM potassium oleate. Enzyme fraction containing the 45 kDa acyl-CoA synthetase was incubated in the reaction mixture containing all the ingredients but coenzyme A for 2 minutes at 37°C. Coenzyme A was then added to initiate the reaction. Reactions were stopped at 3 minutes by removing a 100 μl aliquot from the reaction mixture and put to 500 μl of Dole solution (isopropyl alcohol/heptane/1M sulphuric acid, 40:10:1, v/v/v). An aliquot (300 μl) of water, followed by another 300 μl of heptane were also added. The whole mixture was subjected to vigorous vortexing for 5 seconds. The two phases were allowed to separate. Most of the upper organic phase containing the unreacted radioactive oleic acid was removed by suction. The lower aqueous phase was extracted 3 more times with diethyl ether. The radioactivity remaining was determined by liquid scintillation counting. Background values in control reactions which were carried out under identical conditions with all the ingredients except coenzyme A were subtracted from the experimental values.

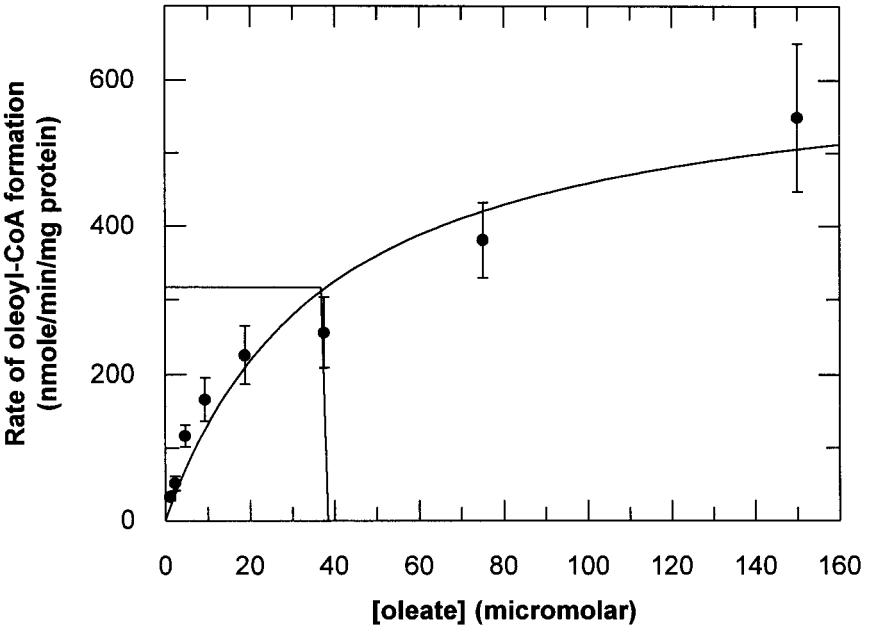

Figure 22. A plot of the initial rate versus the substrate concentration for the 62 kDa acyl-CoA synthetase.

K_M and V_{max} were determined to be 38 ± 12 μM and 633 ± 79 nmole/min/mg protein respectively by fitting data directly into the Michaelis-Menten equation. Each points represented the average of a triplicate, and error bars were ± S.D.. The reaction mixture contained 200mM Tris-Cl, pH 7.5, 16mM $MgCl_2$, 10mM β-mercaptoethanol, 20mM NaF, 0.1% Triton X-100, 10mM ATP, 2.5mM coenzyme A, and 75μM potassium oleate. Enzyme fraction containing the 62 kDa acyl-CoA synthetase was incubated in the reaction mixture containing all the ingredients but coenzyme A for 2 minutes at 37°C. Coenzyme A was then added to initiate the reaction. Reactions were stopped at 3 minutes by removing a 100 μl aliquot from the reaction mixture and put to 500 μl of Dole solution (isopropyl alcohol/heptane/1M sulphuric acid, 40:10:1, v/v/v). An aliquot (300 μl) of water, followed by another 300 μl of heptane were also added. The whole mixture was subjected to vigorous vortexing for 5 seconds. The two phases were allowed to separate. Most of the upper organic phase containing the unreacted radioactive oleic acid was removed by suction. The lower aqueous phase was extracted 3 more times with diethyl ether. The radioactivity remaining was determined by liquid scintillation counting. Background values in control reactions which were carried out under identical conditions with all the ingredients except coenzyme A were subtracted from the experimental values.

3.7.1 Triton X-100 activates both forms of acyl-CoA synthetase

Activities of both the 45 kDa and the 62 kDa acyl-CoA synthetase were minimal in the absence of Triton X-100. However, both forms of the enzyme were activated by 0.1% Triton X-100 (Figures 23 and 24). This supports the previous suggestion that surface is required for optimal activity (Mangroo and Gerber, 1993). In addition, it is now known that both forms of acyl-CoA synthetase have the same requirement of surfaces for activities.

Triton X-100 has a critical micelle concentration of 0.015%. Therefore, in the presence of 0.1% Triton X-100, it is a necessary consequence that the free oleate concentration would be lower than in its absence (Anel *et al.*, 1993). This is because a significant portion of the oleate would partition into the Triton X-100 micelles. In fact, it has been shown that most cis fatty acids would partition into plasma membrane when added to cells (Anel *et al.*, 1993). So, unless acyl-CoA synthetase interacts somehow with the micelles, it will have a lower level of activity due to a lower free oleate concentration.

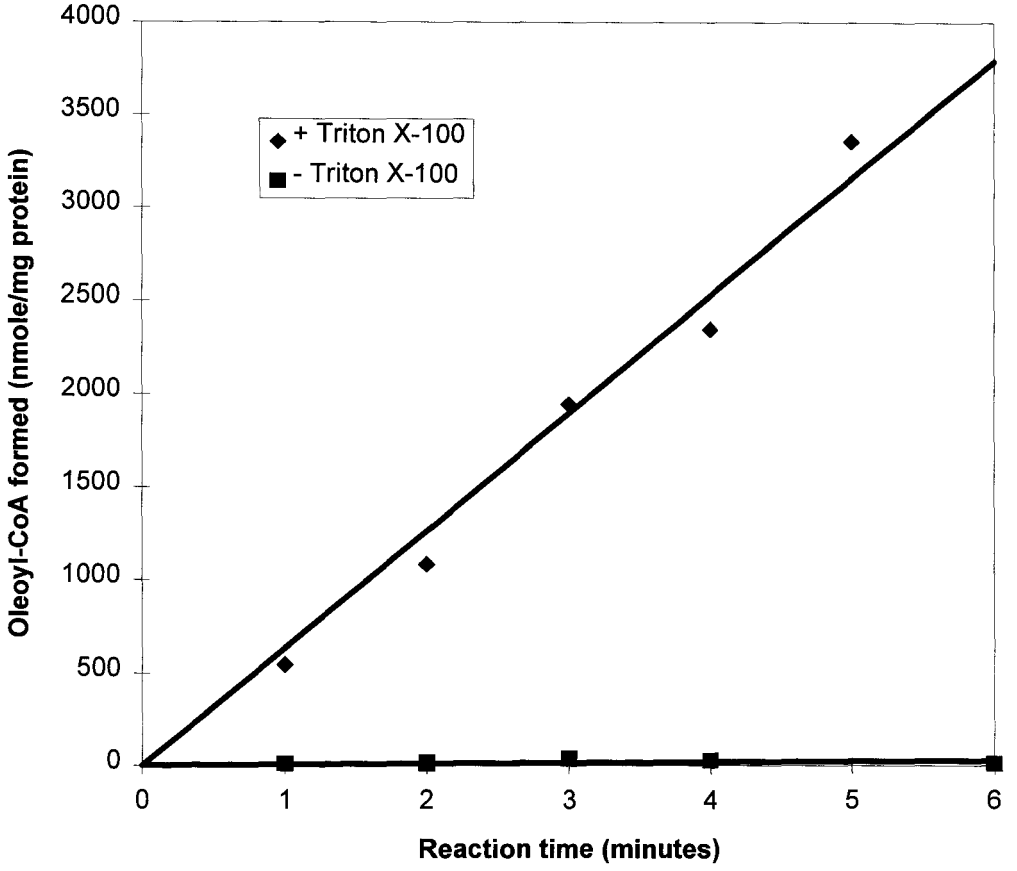

Figure 23. Effect of Triton X-100 on the 45 kDa acyl-CoA synthetase activity.

Reaction time course of the 45 kDa acyl-CoA synthetase in the presence (◆), or absence (■) of 0.1% Triton X-100 (w/v). Oleoyl-CoA formation was monitored by the standard acyl-CoA synthetase activity assay as described in Section 2.7. The reaction mixture contained 200mM Tris-Cl, pH 7.5, 16mM $MgCl_2$, 10mM β-mercaptoethanol, 20mM NaF, 10mM ATP, 2.5mM coenzyme A, and 75μM potassium oleate. Enzyme fraction containing the 45 kDa acyl-CoA synthetase was incubated in the reaction mixture containing all the ingredients but coenzyme A for 2 minutes at 37°C. Coenzyme A was then added to initiate the reaction. Reactions were stopped at the specified time by removing a 100 μl aliquot from the reaction mixture and put to 500 μl of Dole solution (isopropyl alcohol/heptane/1M sulphuric acid, 40:10:1, v/v/v). An aliquot (300 μl) of water, followed by another 300 μl of heptane were also added. The whole mixture was subjected to vigorous vortexing for 5 seconds. The two phases were allowed to separate. Most of the upper organic phase containing the unreacted radioactive oleic acid was removed by suction. The lower aqueous phase was extracted 3 more times with diethyl ether. The radioactivity remaining was determined by liquid scintillation counting. Background values in control reactions which were carried out under identical conditions with all the ingredients except coenzyme A were subtracted from the experimental values.

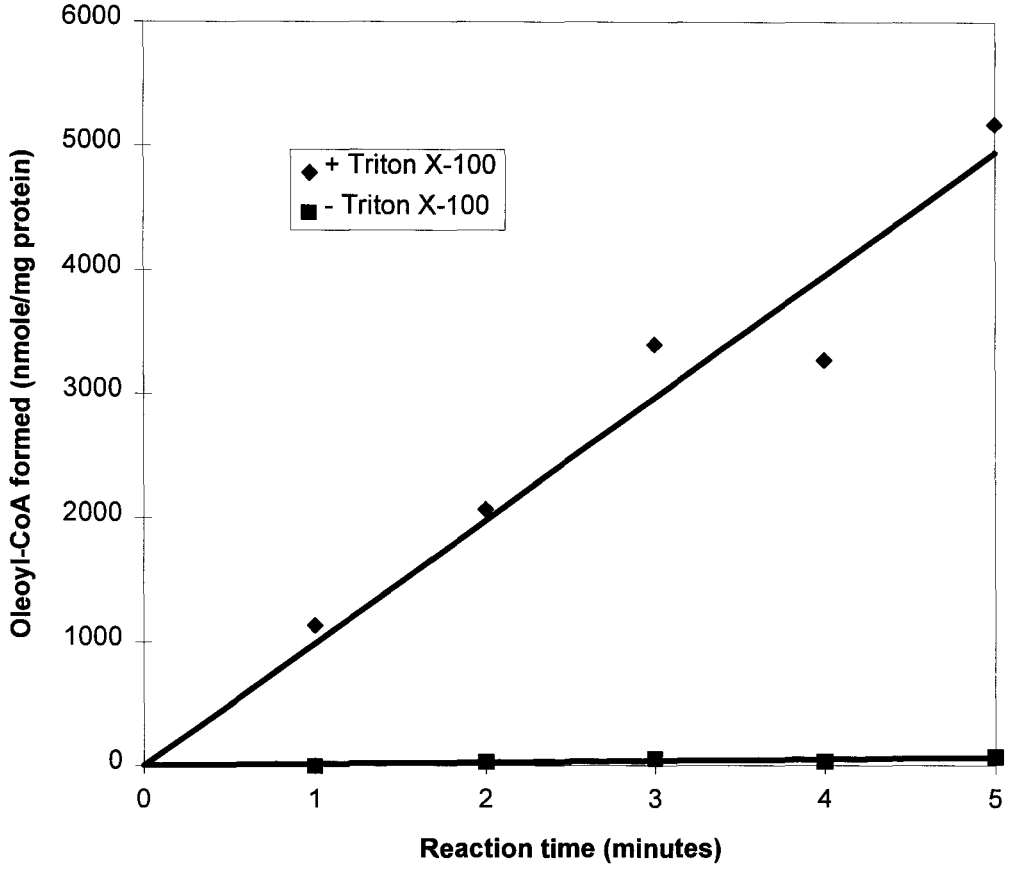

Figure 24. Effect of Triton X-100 on the 62 kDa acyl-CoA synthetase activity.

Reaction time course of the 62 kDa acyl-CoA synthetase in the presence (◆), or absence (■) of 0.1% Triton X-100 (w/v). Oleoyl-CoA formation was monitored by the standard acyl-CoA synthetase activity assay as described in Section 2.7. The reaction mixture contained 200mM Tris-Cl, pH 7.5, 16mM $MgCl_2$, 10mM β-mercaptoethanol, 20mM NaF, 10mM ATP, 2.5mM coenzyme A, and 75μM potassium oleate. Enzyme fraction containing the 62 kDa acyl-CoA synthetase was incubated in the reaction mixture containing all the ingredients but coenzyme A for 2 minutes at 37°C. Coenzyme A was then added to initiate the reaction. Reactions were stopped at the specified time by removing a 100 μl aliquot from the reaction mixture and put to 500 μl of Dole solution (isopropyl alcohol/heptane/1M sulphuric acid, 40:10:1, v/v/v). An aliquot (300 μl) of water, followed by another 300 μl of heptane were also added. The whole mixture was subjected to vigorous vortexing for 5 seconds. The two phases were allowed to separate. Most of the upper organic phase containing the unreacted radioactive oleic acid was removed by suction. The lower aqueous phase was extracted 3 more times with diethyl ether. The radioactivity remaining was determined by liquid scintillation counting. Background values in control reactions which were carried out under identical conditions with all the ingredients except coenzyme A were subtracted from the experimental values.

3.7.2 Acyl-CoA synthetase activity dependence on Triton X-100 concentration

Further investigations on the surface requirement of acyl-CoA synthetase activity involve elucidating the activity dependence on Triton X-100 concentration. It is interesting that the activating effect of Triton X-100 peaked at around 0.0125% Triton X-100 (Figure 25), which is around the critical micelle concentration for Triton X-100. At Triton X-100 concentration lower than 0.0125%, minimal activation was observed. This may be due to the absence of a surface at this concentration range. The activating effect also starts to diminish at Triton X-100 concentration higher than 0.0125%. This may be due to surface dilution of oleate. However, the drop in activation effect plateaued at around 0.1% Triton X-100 (Figure 25), this could not be accounted for simply by the surface dilution of the substrate oleate.

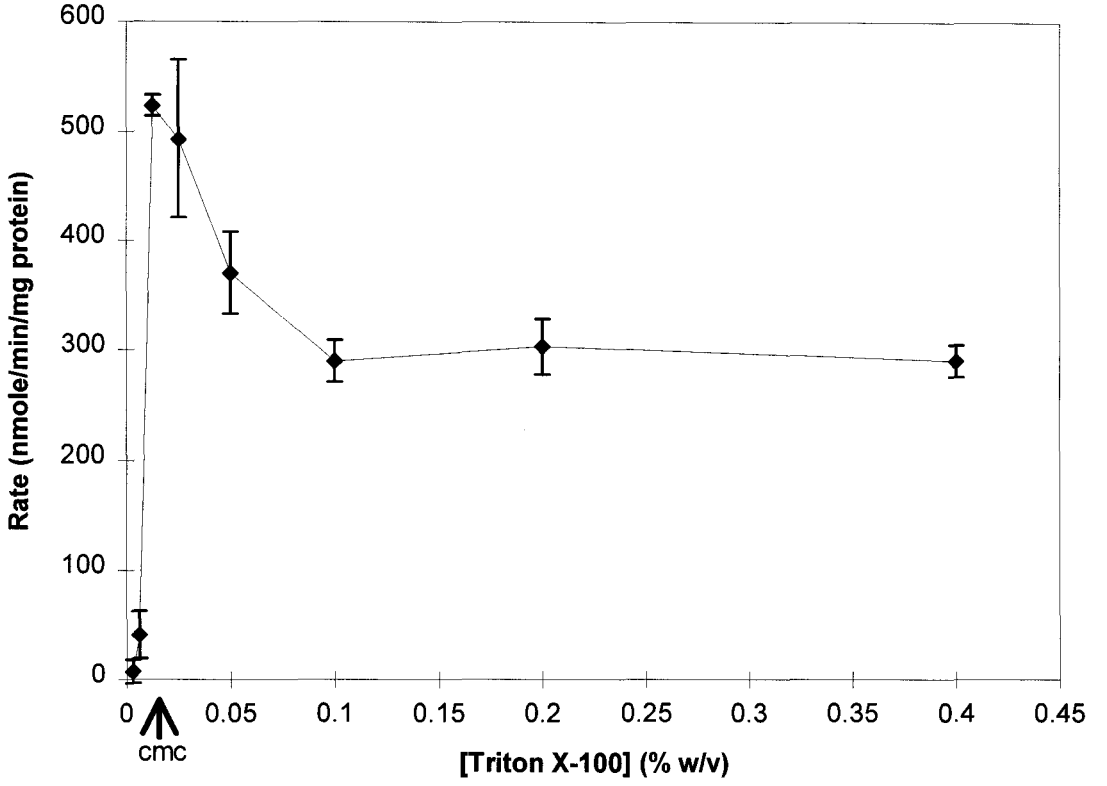

Figure 25. Dependence of the 45 kDa acyl-CoA synthetase acivity on Triton X-100 concentration.

Acyl-CoA synthetase assays were carried out in the presence of 75 μM [9,10-^{3}H] oleate with varying Triton X-100 concentration. The arrow indicates the critical micelle concentration (cmc) of Triton X-100. Each data point is an average of triplicate, and the error bars are ± S.D.. The reaction mixture contained 200mM Tris-Cl, pH 7.5, 16mM $MgCl_2$, 10mM β-mercaptoethanol, 20mM NaF, 10mM ATP, 2.5mM coenzyme A, and 75μM potassium oleate. Enzyme fraction containing the 45 kDa acyl-CoA synthetase was incubated in the reaction mixture containing all the ingredients but coenzyme A for 2 minutes at 37°C. Coenzyme A was then added to initiate the reaction. Reactions were stopped at 3 minutes by removing a 100 μl aliquot from the reaction mixture and put to 500 μl of Dole solution (isopropyl alcohol/heptane/1M sulphuric acid, 40:10:1, v/v/v). An aliquot (300 μl) of water, followed by another 300 μl of heptane were also added. The whole mixture was subjected to vigorous vortexing for 5 seconds. The two phases were allowed to separate. Most of the upper organic phase containing the unreacted radioactive oleic acid was removed by suction. The lower aqueous phase was extracted 3 more times with diethyl ether. The radioactivity remaining was determined by liquid scintillation counting. Background values in control reactions which were carried out under identical conditions with all the ingredients except coenzyme A were subtracted from the experimental values.

Alternatively, the surface dilution effect may be on the surface charge of the micelles. The cytoplasmic membrane in *Escherichia coli* is negatively charged, however, the Triton X-100 micelle is electrically neutral. When oleate partitioned into the micelles, it provided an overall negative charge to the micelle. However, as more and more Triton X-100 was added to the reaction mixture, the less negative charge would be per unit of micelle surface area because the amount of oleate in the system was fixed. It is possible that the negative surface charge contributed by the oleate at higher Triton X-100 concentration was not sufficient to affect the acyl-CoA synthetase activity. This is not to say that the surface was no longer useful, just that the surface was no longer carrying a useful surface charge for additional stimulation. In fact, *Escherichia coli* phospholipids contains around 70% to 80% phosphatidylethanolamine, 15% to 25% phosphatidylglycerol, and 5% to 10% cardiolipin (Kadner, 1996). Phosphatidylglycerol carries one net negative charge, cardiolipin carries two net negative charges, while phosphatidylethanolamine is zwitterionic. This charge ratio is similar to that when the oleate mole fraction is at 0.3, at which maximal activation was observed (Figure 26).

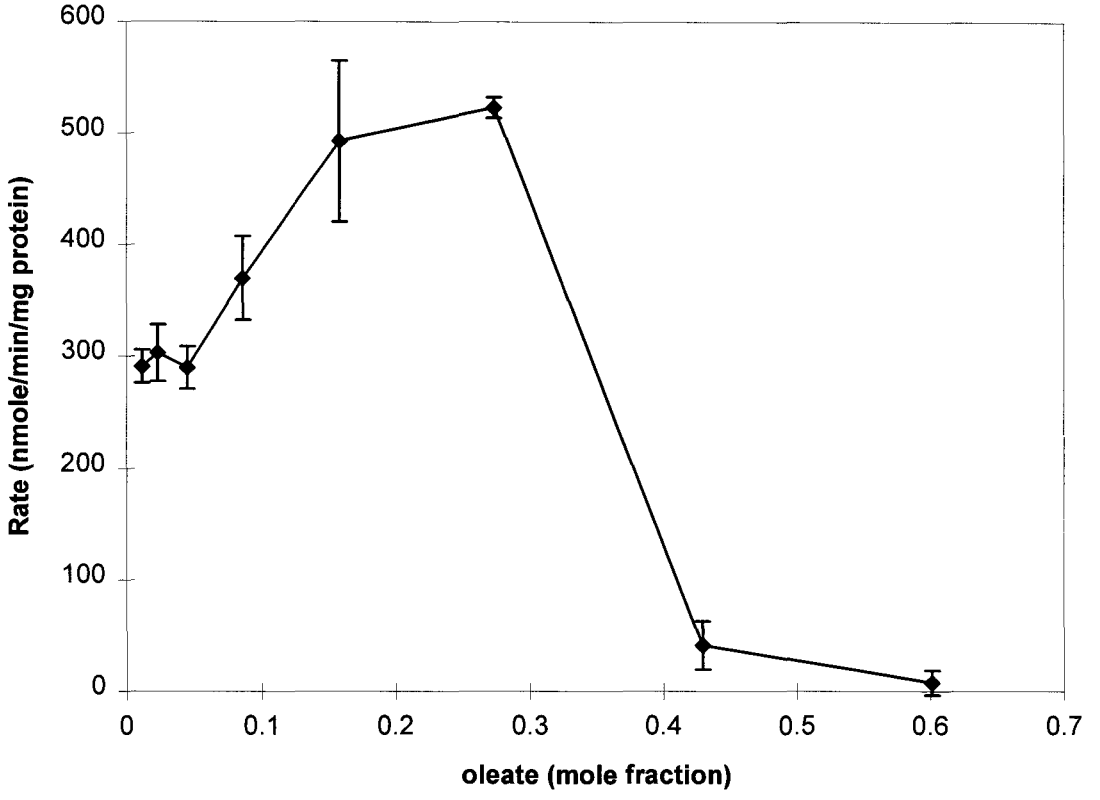

Figure 26. Dependence of the 45 kDa acyl-CoA synthetase activity on Triton X-100 concentration expressed as oleate mole fraction.

Acyl-CoA synthetase assays were carried out in the presence of 75 μM [9,10-^{3}H] oleate with varying Triton X-100 concentration. Each data point is an average of triplicate, and the error bars are ± S.D.. The reaction mixture contained 200mM Tris-Cl, pH 7.5, 16mM $MgCl_2$, 10mM β-mercaptoethanol, 20mM NaF, 10mM ATP, 2.5mM coenzyme A, and 75μM potassium oleate. Enzyme fraction containing the 45 kDa acyl-CoA synthetase was incubated in the reaction mixture containing all the ingredients but coenzyme A for 2 minutes at 37°C. Coenzyme A was then added to initiate the reaction. Reactions were stopped at 3 minutes by removing a 100 μl aliquot from the reaction mixture and put to 500 μl of Dole solution (isopropyl alcohol/heptane/1M sulphuric acid, 40:10:1, v/v/v). An aliquot (300 μl) of water, followed by another 300 μl of heptane were also added. The whole mixture was subjected to vigorous vortexing for 5 seconds. The two phases were allowed to separate. Most of the upper organic phase containing the unreacted radioactive oleic acid was removed by suction. The lower aqueous phase was extracted 3 more times with diethyl ether. The radioactivity remaining was determined by liquid scintillation counting. Background values in control reactions which were carried out under identical conditions with all the ingredients except coenzyme A were subtracted from the experimental values.

3.8 Conclusions

The starvation effect of reducing oleate uptake rate in *Escherichia coli* was only observed in the initial minute post starvation. This starvation effect was not due to the depletion of its substrates ATP or reduced coenzyme A. Neither was the effect caused by a lowered acyl-CoA synthetase level. This reduction in oleate uptake rate can be reversed by incubation in oleate. It was also discovered that when [9,10-^{3}H]oleate was used as the radioactive tracer, the majority of the radioactivity was effluxed from the cells. It was found that lactate did not have an effect on the overall oleate uptake. Its apparent activation on oleate uptake was due to a reduction in the efflux rate of radioactive probes, instead of an increase in the uptake rate of oleate.

A new form of acyl-CoA synthetase was partially purified. This form, which had a size of around 62 kDa, could be converted to its 45 kDa counterpart in a Triton X-100 and temperature dependent manner. This induced processing of the 62 kDa enzyme was found to be inhibited by oleate, which is a natural substrate of acyl-CoA synthetase. The two forms of acyl-CoA synthetase were shown to be immunologically related.

A much simpler purification scheme was presented for the 45 kDa acyl-CoA synthetase by using the membrane bound form of acyl-CoA synthetase as starting materials. The availability of an over-expression system, the selective extraction of the 62 kDa acyl-CoA synthetase from the membrane, together with

the induced processing into the 45 kDa form had provided the basis for this comparatively simple purification process.

The kinetics parameters of both forms of the acyl-CoA synthetase were determined under standard assay conditions. The K_M and V_{max} for the 45 kDa acyl-CoA synthetase were determined to be 85 ± 18 μM and 1550 ± 165 nmole/min/mg protein respectively, whereas the K_M and V_{max} for the 62 kDa enzyme were determined to be 38 ± 12 μM and 633 ± 79 nmole/min/mg protein respectively. This suggests that the 62 kDa enzyme has higher affinity towards oleate than its 45 kDa counterpart, at least under standard assay conditions. However, the ratio between the V_{max} and the K_M remained the same.

Evidence suggesting Triton X-100 activates acyl-CoA synthetase by providing a surface for catalysis was presented. This interaction between acyl-CoA synthetase and the Triton X-100 / oleate mixed micelle may be affected by the surface charge of the mixed micelle.

3.9 Future Investigations

The mechanism of how lactate inhibits the efflux of radioactivity remains unclear. There is likely a regulatory mechanism that controls the level of β-oxidation at different physiological states of the cells. It is possible that lactate, which is an energy source for generating the membrane potential, interacts with fatty acid β-oxidation which also generates membrane potential. Since, the membrane potential has to be dynamically maintained at a certain controlled

level, in the presence of lactate, β-oxidation would occur at a lower rate, possibly because of a slower regeneration of FAD in acyl CoA dehydrogenase and/or NAD^+ due to a lower abundance of electron acceptors (e.g., oxidized form of CoQ) resulting from competition with D-lactate. It would be interesting to find out why the *tsp*⁻ mutant failed to respond to the inhibitory effect of lactate on radioactivity efflux. Since membrane potential may have a role in regulating β-oxidation, then, is it possible that the *tsp*⁻ mutant lacks the ability to regulate the membrane potential, e.g. fail to slow down β-oxidation in response to alternate substrates or hyper potential, as in wild type *Escherichia coli*?

Although two forms of acyl-CoA synthetase were identified, are both of them present *in vivo*? Acyl-CoA synthetase has been described as both cytosolic and peripheral in the literature. However, these descriptions are based on the activity distribution between the cytosolic and the membrane fraction during purification, i.e., after cells have been disrupted. Therefore, there has not been any observation made on the presence of the 45 kDa acyl-CoA synthetase *in vivo*. If it is present, then is the distribution between the two forms governed by some unknown factors, e.g. starvation, fatty acid level? It has been reported that in the presence of lactate, acyl-CoA synthetase activity would be retained in the membrane fraction despite extensive washing (Mangroo and Gerber,1993). On the other hand, it was observed in the present study that the 62 kDa acyl-CoA synthetase binds very tightly to the membrane, while no observable amount

of the 45 kDa acyl-CoA synthetase is seen in the membrane fraction. Therefore, it is possible that lactate caused recruitment of acyl-CoA synthetase to the membrane by preventing the processing of the 62 kDa acyl-CoA synthetase to the 45 kDa form. Also, since lactate had to be added to the cell lysing buffer in the beginning of the procedure to be effective, is it possible then that lactate prevents the processing of acyl-CoA synthetase by acting as an alternate energy source and hence preserving the carry over oleate from being metabolized?

The site of acyl-CoA synthetase catalysis *in vivo* is not yet known. Suggestive evidence was presented in the present study that a membranous surface is required for acyl-CoA synthetase activity *in vitro*, and it follows that the enzyme works on the surface of the membrane, i.e., interfacial catalysis. It would be interesting to show that the acyl-CoA synthetase can be recruited to the cytoplasmic membrane *in vivo*. This can be done by photochemically cross-linking all membrane bound enzymes to [^{32}P] photoreactive phospholipids in the membrane *in vivo*, followed by the isolation of the membrane fraction and immuno-detection of any acyl-CoA synthetase that is cross-linked to [^{32}P] phospholipids (Gerber, personal communication). This allows investigations on membrane recruitment conditions *in vivo*. This technique, once developed, can also be applied to studies involving other membrane recruitable enzymes, as long as specific antibodies are available to those enzymes.

Scaling up and improving the purification process would allow the initiation of crystallization trials on acyl-CoA synthetase. The ultimate goal is to have the structure of the enzyme resolved. This allows the structural and functional analysis to be performed on this potentially membrane recruitable enzyme.

4. REFERENCES

Anel, A., Richieri, G.V., and Kleinfeld, A.M. (1993) Membrane Partition of Fatty Acids and Inhibition of T Cell Function. Biochemistry **32**, 530-536.

Azizan, A. and Black, P. N. (1994) Use of Transposon Tn*phoA* To Identify Genes for Cell Envelope Proteins of *Escherichia coli* Required for Long-Chain Fatty Acid Transport: the Periplasmic Protein Tsp Potentiates Long-Chain Fatty Acid Transport. Journal of Bacteriology **176**, 6653-6662.

Bagnara, A. S. and Finch, L. R. (1972) Quantitative Extraction and Estimation of Intracellular Nucleoside Triphosphates of *Escherichia coli.* Analytical Biochemistry **45**, 24-34.

Bar-Tana, J., Rose, G., and Shapiro, B. (1971) The purification and properties of microsomal palmitoyl-coenzyme A synthetase. Biochem. J. **122**, 353-362.

Benson, R. W., Robinson, J. L. and Boyer, P. D. (1969) Quantitative Appraisals of Possible Catalytic Intermediates in the Succinyl Coenzyme A Synthetase Reaction. Biochemistry **8**, 2496-2502.

Black, P. N. (1988) The *fadL* Gene Product of *Escherichia coli* Is an Outer Membrane Protein Required for Uptake of Long-Chain Fatty Acids and Involved in Sensitivity to Bacteriophage T2. Journal of Bacteriology **170**, 2850-2854.

Black, P. N. and DiRusso, C. C. (1994) Molecular and biochemical analyses of fatty acid transport, metabolism, and gene regulation in *Escherichia coli.* Biochimica et Biophysica Acta **1210**, 123-145.

Black, P.N., DiRusso, C.C., Metzger, A.K., and Heimert, T.L. (1992) Cloning, Sequencing, and Expression of the fadD Gene of *Escherichia coli* Encoding Acyl Coenzyme A Synthetase. Journal of Biological Chemistry **267**, 25513-25520.

Black, P.N. and Zhang, Q. (1995) Evidence that His^{110} of the protein FadL in the outer membrane of *Escherichia coli* is involved in the binding and uptake of long-chain fatty acids: possible role of this residue in carboxylate binding. Biochemical Journal **310**, 389-394.

Corkey, B. E. (1988) Analysis of Acyl-Coenzyme A Esters in Biological Samples. Methods in Enzymology **166**, 55-70.

Clark, D. P. and Cronan, J. E. (1996) Two-Carbon Compounds and Fatty Acids as Carbon Sources. *In* Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E. (ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd Ed. ASM press, Washington, D.C..

Cronan, J. E. and LaPorte, D. (1996) Tricarboxylic Acid Cycle and Glyoxylate Bypass. *In* Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E. (ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd Ed. ASM press, Washington, D.C..

DeBuysere, M. S. and Olson, M. S. (1983) The Analysis of Acyl-coenzyme A Derivatives by Reverse-Phase High-Performance Liquid Chromatography. Analytical Biochemistry **133**, 373-379.

DiRusso, C.C., Heimert, T. L., and Metzger, A. K. (1992) Characterization of FadR, a Global Transcriptional Regulator of Fatty Acid Metabolism in *Escherichia coli* ; Interaction with the *fadB* promotor is prevented by long chain fatty acyl coenzyme A's. Journal of Biological Chemistry **267**, 8685-8691.

Fulda, M., Heinz, E. and Wolter, F. P. (1994) The *fadD* gene of *Escherichia coli* K12 is located close to *rnd* at 39.6 min of the chromasomal map and is a new member of the AMP-binding protein family. Molecular and General Genetics **242**, 241-249.

Jackowski, S. and Rock, C. O. (1986) Consequences of Reduced Intracellular Coenzyme A Content in *Escherichia coli.* Journal of Bacteriology **166**, 866-871.

Joshi, A. K., Ahmed, S. and Ames, G. F. L. (1989). Energy coupling in bacterial periplasmic transport systems: Studies in intact *Escherichia coli* cells. Journal of Biological Chemistry **264**, 2126-2133.

Kadner, R. J. (1996) Cytoplasmic Membrane, p. 58-87. *In* Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E. (ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd Ed. ASM press, Washington, D.C..

Kameda, K. and Nunn, W.D. (1981) Purification and Characterization of Acyl Coenzyme A Synthetase from *Escherichia coli*. Journal of Biological Chemistry **256**, 5702-5707.

Kameda, K., Suzuki, L. K., and Imai, Y. (1985) Futher purification, characterization and salt activation of acyl-CoA synthetase from *Escherichia coli*. Biochimica et Biophysica Acta **840**, 29-36.

Klein, K., Steinberg, R., Fiethen, B. snd Overath, P. (1971) Fatty acid degradation in *Escherichia coli*: An Inducible System for the Uptake of Fatty Acids and further Characterization of *old* Mutants. European Journal of Biochemistry **19**, 442-450.

Kumar, G. B. and Black, P. N. (1993) Bacterial Long-chain Fatty Acid Transport; Identification of amino acid residues within the outer membrane protein FadL required for activity. Journal of Biological Chemistry **268**, 15469-15476.

Laemmli, U. K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature **227**, 680-685.

Maloy, S. R., Ginsburgh, C. L., Simons, R. W., and Nunn, W. D. (1981) Transport of Long and Medium Chain Fatty Acids by *Escherichia coli* K12. Journal of Biological Chemistry **256**, 3735-3742.

Mangroo, D. (1992) Fatty Acid Uptake in *Escherichia coli*: Development of a Photoaffinity Labeling Approach for Identifying Proteins Involved in the Transmembrane Movement of Long Chain Fatty Acids. Ph.D. Thesis, McMaster University.

Mangroo, D. and Gerber, G. E. (1992) Photoaffinity Labeling of Fatty Acid-binding Proteins Involved in Long Chain Fatty Acid Transport in *Escherichia coli*. Journal of Biological Chemistry **267**, 17095-17101.

Mangroo, D. and Gerber, G. E. (1993) Fatty acid uptake in *Escherichia coli* : regulation by recruitment of fatty acyl-CoA synthetase to the plasma membrane. Biochemistry and Cell Biology **71**, 51-56.

Nikaido, N. and Vaara, M. (1985) Molecular Basis of Bacterial Outer Membrane Permeability. Microbiological Reviews **49**, 1-32.

Nunn, W. D. (1986) A Molecular View of Fatty Acid Catabolism in *Escherichia coli*. Microbiological Reviews **50**, 179-192.

O'Brien, W.J. and Frerman, F.E. (1980) The Effect of Temperature and Membrane Lipid Composition on the Rate of β-oxidation by *Escherichia coli*. Biochimica et Biophysica Acta **617**, 20-27.

Oliver, D. B. (1996) Periplasm, p. 88-103. *In* Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E. (ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd Ed. ASM press, Washington, D.C..

Overath, P., Pauli, G., and Schairer, H. U. (1969) Fatty Acid Degradation in *Escherichia coli*: An Inducible Acyl-CoA Synthetase, the Mapping of *old*-Mutations, and the Isolation of Regulatory Mutants. European Journal of Biochemistry **7**, 559-574

Ramirez, F. and Jain, M. K. (1991) Phospholipase A_2 at the Bilayer Interface. Proteins: Structure, Function, and Genetics **9**, 229-239.

Samuel, D., Estroumza, J., and Ailhaud, G. (1970) Partial Purification and Properties of Acyl-CoA Synthetase of *Escherichia coli*. European Journal of Biochemistry **12**, 576-582.

Silbert, D.F., Cohen, M., and Harder, M.E. (1972) The Effect of Exogenous Fatty Acid Metabolism is *Escherichia coli* K-12. Journal of Biological Chemistry **247**, 1699-1707.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and some Applications. Proc. Natl. Acad. Sci. U.S.A. **76**, 4350-4354.

Vallari, D. S. and Jackowski, S. (1988) Biosynthesis and Degradation Both Contribute to the Regulation of Coenzyme A Content in *Escherichia coli* . Journal of Bacteriology **170**, 3961-3966.