LIPOAMIDASE FROM *Enterococcus faecalis*

By

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This thesis describes work aimed at purifiying lipoamidase from *Enterococcus faecalis*. Lipoamidase is the enzyme which hydrolyzes the amide bond between liopic acid and a lysine residue on the core subunit of the α-keto acid dehydrogenase complexes. The cloning of the lipoamidase gene will facilitate the study of lipoic acid function in a number of systems.

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LIST OF ABBREVIATIONS

ABI	Applied Biosystems Incorporated
apoPDH	Inactive PDH without lipoic acid attached
APS	Ammonium Persulfate
BSA	Bovine Serum Albumin
CDNB	1-chloro-2,4-dinitrobenzene
CHT	Bio-Rad hydroxylapatite column
CoA	Coenzyme A
DEAE	Diethylaminoethyl
DE	Designation for a DEAE fraction
DTNB	5,5'-dithiobis(2-nitrobenzoate)
E1	Pyruvate decarboxylase subunit of PDH
E2	Dihydrolipoyl transacetylase subunit of PDH
E3	Dihydrolipoyl dehydrogenase subunit of PDH
EF-Ts	Elongation Factor Ts
EF-Tu	Elongation Factor Tu
EtOH	Ethanol
FAD	Flavin Adenine Dinucleotide (oxidized)
$FADH_2$	Flavin Adenine Dinucleotide (reduced)
Flow through	Protein fraction from column chromatography which is not retained
GSH	Glutathione
GST	Glutathione S-transferase
GST-LipDom	Glutathione S transferase lipoyl domain fusion protein
HIC	Hydrophobic Interaction Chromatography
holoPDH	Complete active PDH with lipoic acid attached
HTP	Hydroxylapatite
IPTG	Isopropyl β-D-thiogalactopyranoside
KCl	Potassium chloride
KPO_4	Potassium phosphate buffer
LA-*p*ABA	N-D,L-Lipoyl p-aminobenzoic Acid
LDH	Lactate dehydrogenase
LipDom	Human lipoyl domain
Liplys	Lipoyl-lysine
LLH	Lipoyl-lysine hydrolase
LPH	Lipoyl-*p*ABA hydrolase
MeOH	Methanol
NAD^+	Nicotinamide Adenine Dinucleotide (oxidized)
NADH	Nicotinamide Adenine Dinucleotide (reduced)
*p*ABA	para-Aminobenzoic Acid
NEDD	N-1-naphthylethylenediamine dichloride
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PDH	Pyruvate Dehydrogenase
PEG	Polyethyleneglycol 6000

pHMB	para-hydroxymercuribenzoate
PMSF	Phenyl-methylsulfonyl chloride
PTA	Phosphotransacetylase
PVDF	Polyvinylidene difluoride
RDL FT	Flo-through from dye affinity chromatography screening
SDS	Sodium Dodecylsulfate
SONS	Sonicated supernatant solution
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
THF	Tetrahydrofuran
TNB	Thionitrobenzoate
TPP	Thiamine Pyrophosphate

CHAPTER I

INTRODUCTION

The major portion of carbon oxidized in biological systems passes through pyruvate dehydrogenase on its way to the citric acid cycle. Pyruvate dehydrogenase requires thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), coenzyme A, nicotinamide adenine dinucleotide (NAD^+) and lipoic acid cofactors. While all of the cofactors are required for pyruvate dehydrogenase function, only lipoic acid requires enzymatic assistance for incorporation and removal from the complex.

A. Lipoic Acid Discovery

Lipoic acid (1,2-dithiolane-3-pentanoic acid or DL-6,8-thioctic acid, also known as protogen A, acetate replacing factor and pyruvate oxidation factor) was first isolated from liver in a cooperative effort by L.J. Reed, I.C. Gunsalus and the Eli Lilly Company in the early 1950's (1). The 8 carbon compound contains a carboxyl group and a thiolane ring that is convertible to a dithiol form with reducing agents. The structures of the interconvertible forms, lipoic acid and dihydrolipoic acid, are shown in Fig. I-1.

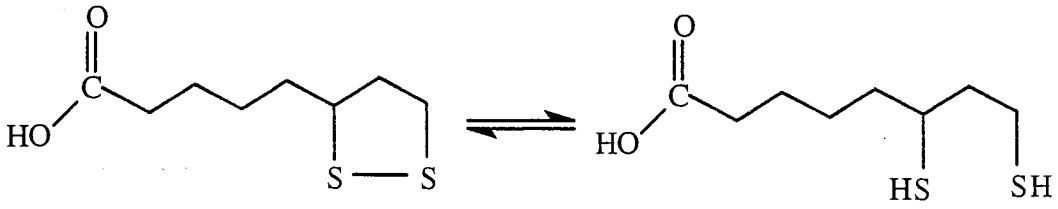


Figure I-1. Lipoic Acid. Shown in its oxidized (left) and reduced (right) form.

By 1957 L.J. Reed *et al.* (2) had presented evidence that protein-bound lipoic acid is involved in the oxidative decarboxylation of α-keto acids by α-keto acid dehydrogenases. Lipoic acid is essential to the function of all α-keto acid dehydrogenases and the glycine cleavage system. In 1960 Nawa *et al.* (3) showed that protein bound lipoic acid is attached by its carboxyl group through an amide linkage to the ε–amino group of a lysine residue as shown in Fig. I-2.

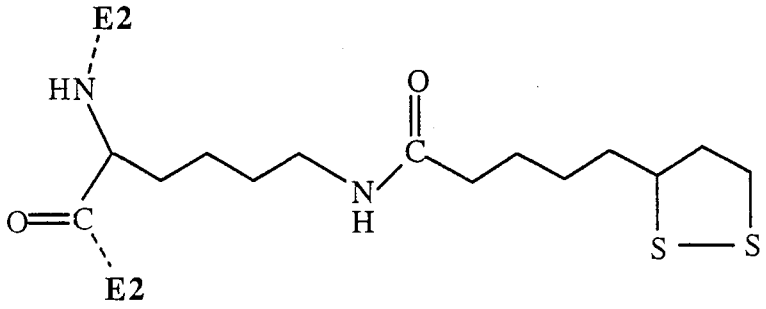


Figure I-2. Protein Bound Lipoic Acid in an Amide Linkage to the ε-NH_2 of Lysine. E2 designates the amino acid backbone of the E2 subunit of α-keto acid dehydrogenases. The structure gives a flexible arm 14 angstroms long.

B. Lipoic Acid Biosynthesis

In *Eschericia coli* lipoic acid is synthesized from octanoyl-ACP (acyl carrier protein) by the addition of sulfur to the number 6 and 8 carbons [Morris *et al.* (4)]. Hayden *et al.* (5, 6) showed that the *E. coli* gene *lipA* is involved in the sulfur insertion and is similar to the system for biotin synthesis. The *lipB* gene, identified by K.E. Reed *et al.* (7) is

involved with lipoic acid synthesis after sulfur insertion. Further work by Morris *et al.* (8) showed *lipB* and another gene, *lplA*, coded for ligases responsible for the attachment of lipoic acid to proteins. These enzymes have substrate specificity differences: *lipB* prefers lipoic acid but will incorporate octanoic acid in the absence of lipoic acid while *lplA* will only use lipoic acid. Siggaard-Anders *et al.* (9) reported that, in *E. coli,* a β-ketoacyl-[ACP] synthase condenses octanoic acid with ACP to supply octanoic acid for lipoic acid biosynthesis. The authors speculated that a similar enzyme would function in plant mitochondria.

In eucaryotes fatty acid synthesis generally occurs in the cytosol. However, Wada *et al.* (10) identified a complete fatty acid synthesis system in pea mitochondria as had been speculated by Siggaard-Anders *et al.* (9). This system uses ACP to make fatty acids of shorter chain length than made by the system found in plastids, and the investigators suggested that most of the fatty acids synthesized by this system are used for lipoic acid biosynthesis. Sulo *et al.* (11) identified the yeast gene *LIP5* by its ability to complement a mutant with a defective lipoic acid synthesis system. The *LIP5* sequence contains a mitochondria targeting sequence in the leader peptide and is homologous to the *lipA* of *E. coli*, which is responsible for the sulfur insertion step. Recently Brody *et al.* (12) reported that the *Saccaromyces cerevisiae* mitochondrial ACP is also involved in synthesis of short chain fatty acids and that an *ACP1* mutant had only 5-10% the normal level of lipoic acid. Jordan and Cronan (13) found that *E. coli* and mitochondria of higher plants can transfer lipoic acid directly from ACP to lipoic acid-dependent enzymes. All of these data indicate that biosynthesis of lipoic acid is a special case of fatty acid biosynthesis. Instead of being synthesized in the cytosol like most fatty acids, lipoic acid is made in the same subcellular compartment as many of the proteins for which it is a vital component.

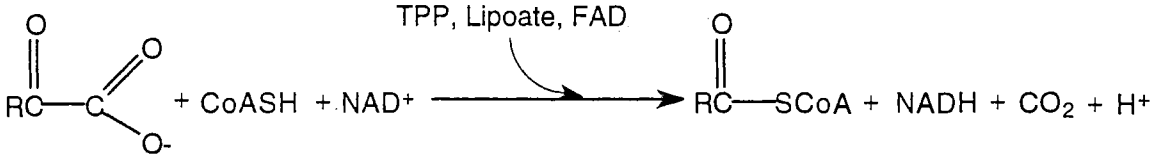
C. Other Lipoic Acid Functions

Lipoic acid is currently receiving attention as a general anti-oxidant and specifically for helping prevent or reduce damage due to reactive oxygen radicals in diabetes, radiation exposure, and cardiac and cerebral ischemia (14). In addition putative lipoyl domains have been found in proteins in which they have as yet no known function (15) .

D. a-Keto Acid Dehydrogenases

The α-keto acid dehydrogenase family of multi-enzyme complexes catalyzes the oxidative decarboxylation of substrate to form an acyl-coenzyme A and NADH. This family has three members, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and the branched chain α-keto acid dehydrogenase. Pyruvate dehydrogenase (PDH) converts pyruvate to acetyl-coenzyme A (acetyl-CoA) for the citric acid cycle, fatty acid biosynthesis and for acetylcholine synthesis. α-Ketoglutarate dehydrogenase converts α-ketoglutarate to succinyl-CoA in the citric acid cycle. The branched chain α-keto acid dehydrogenase utilizes multiple substrates in the degradation of branched chain amino acids.

These multienzyme complexes have at least three components with catalytic activity; an α-keto acid dehydrogenase (E1), a dihydrolipoyl transacylase (E2) and a dihydrolipoyl dehydrogenase (E3). The overall reaction catalyzed by these complexes is:



The E1 subunit decarboxylates the α-keto acid in a thiamine pyrophosphate-dependent (TPP) reaction with the formation of a hydroxyalkyl-TPP intermediate. The hydroxyalkyl-TPP is then oxidized by lipoamide to form an acyl-TPP. The acyl group is transferred to

an E2-lipoamide. The acyl group is then shuttled via multiple lipoamides to the transacylase active site on the E2 subunit where it is finally transferred to CoA through a acyl transfer reaction with the formation of dihydrolipoamide and the release of acyl-CoA. A FAD associated with the E3 subunit then regenerates the lipoamide and is in turn reoxidized by NAD^+ (Fig. I-3). The lipoamide of E2 is lipoic acid attached to the ε-amino group of lysine forming a 14 Angstrom long flexible swinging arm.

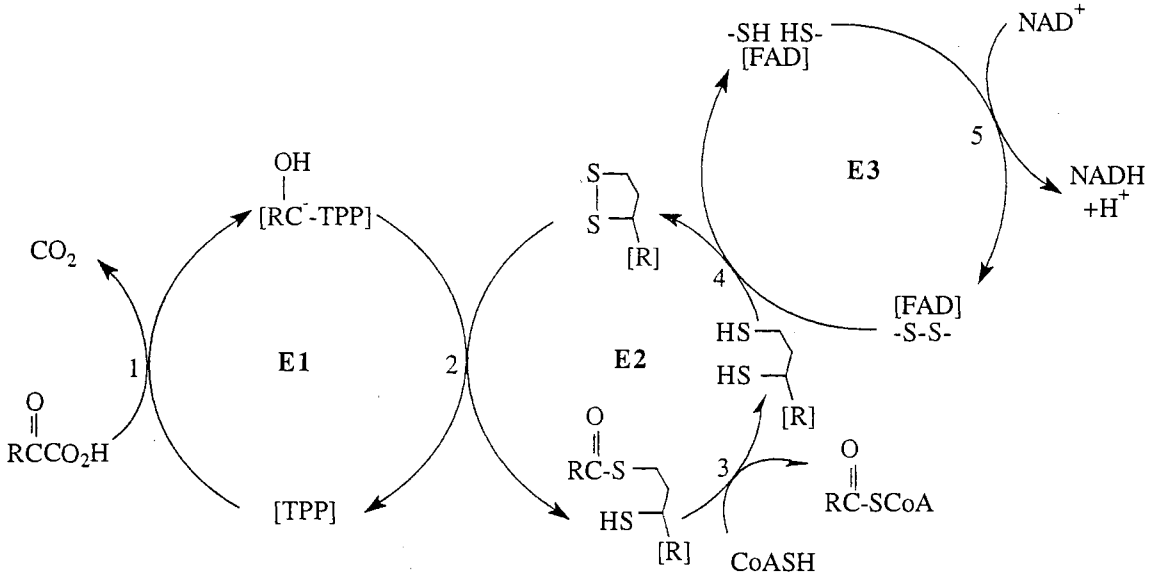


Figure I-3. Reactions of the α-Keto Acid Dehydrogenases. Five reactions are carried out by three enzyme subunits resulting in one acyl-CoA and one NADH generated per α-keto acid molecule reacted.

E. Regulation

Procaryotic complexes are controlled by product inhibition by both NADH and acyl-CoA. There is no kinase or phosphatase component in procaryotic α-keto acid dehydrogenase regulation.

The eucaryotic dehydrogenase complexes have, in addition to E1, E2, and E3, an X subunit which has a lipoyl domain. Recently X was designated E3BP (E3 binding protein) (16) as it is involved in the contact between the E2 core and E3 dimers. The lipoyl moieties of X may have a role in the catalytic activity of the complex and X is involved with

regulation of the complexes through interactions with a kinase (16). Since eucaryotic α-keto acid dehydrogenases are regulated by phosphorylation/dephosphorylation there are kinase and phophatase requirements (16).

F. Structure

The α-keto acid dehydrogenases are very large self-assembling complexes composed of multiple copies of the three subunits. The E2 subunits form the core of the complex. In *E. coli* there are 24 E2 subunits around which 12 E3 homodimers and 12 E1 monomers associate forming a ~4600 kDa complex (Fig. I-4) (17).

Using trypsin and lipoamidase on *E. coli* α-keto dehydrogenases Stepp *et al.* (18) discovered the domain nature of the E2 subunit. Treatment of the complex with trypsin revealed a sensitive flexible region in the E2. Cleavage at this flexible region released polypeptides with lipoic acid attached whereas treatment with lipoamidase released only lipoic acid. Interestingly about half of the lipoyl domains in the complex could be released with no significant loss of overall dehydrogenase activity. Later work using trypsin and *Staphylococcus aureus* V8 proteinase and then sequencing of the released peptides showed that cleavage occurred in stretches of the protein which are rich in alanine, proline and charged amino acids (19). These stretches have conformational flexibility and are probably involved in lipoyl domain movement. The *E. coli* PDH can assemble and carry out full catalytic activity after deletion of two of its three lipoyl domains (20) from the E2 subunits. These results indicate a complex mechanism for the shuttling of acyl groups among active sites. By using a computer modeling system with *E. coli* complex data Hackert *et al.* (21) calculated that two lipoyls are available at each E1 active site and suggested a lipoyl-lipoyl transfer network that shuttles electrons and acyl groups around the complex. Figure I-5 shows a schematic representation of the lipoyl-lipoyl transfer network.

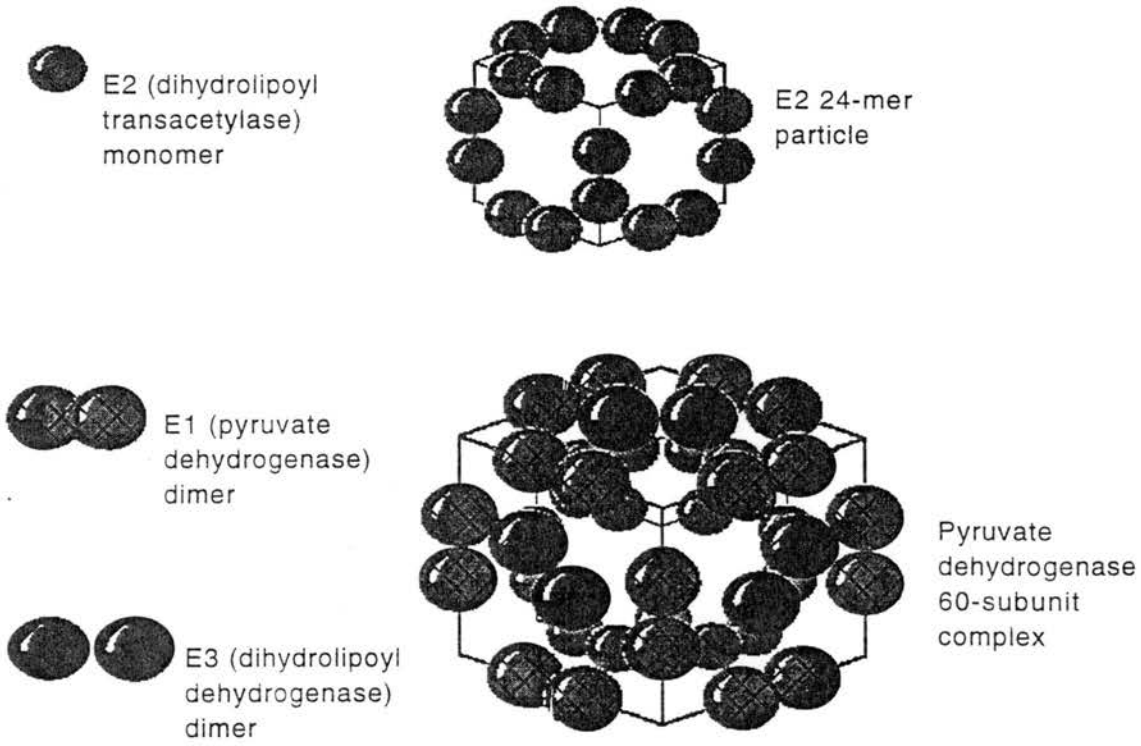


Figure I-4. PDH Subunit Arrangement. (Diagram was downloaded from www.biochemtech.uni-halle.de/PPS2/course/section11/pdhcomp.gif. It was adapted from Voet and Voet (1990) after Lester Reed).

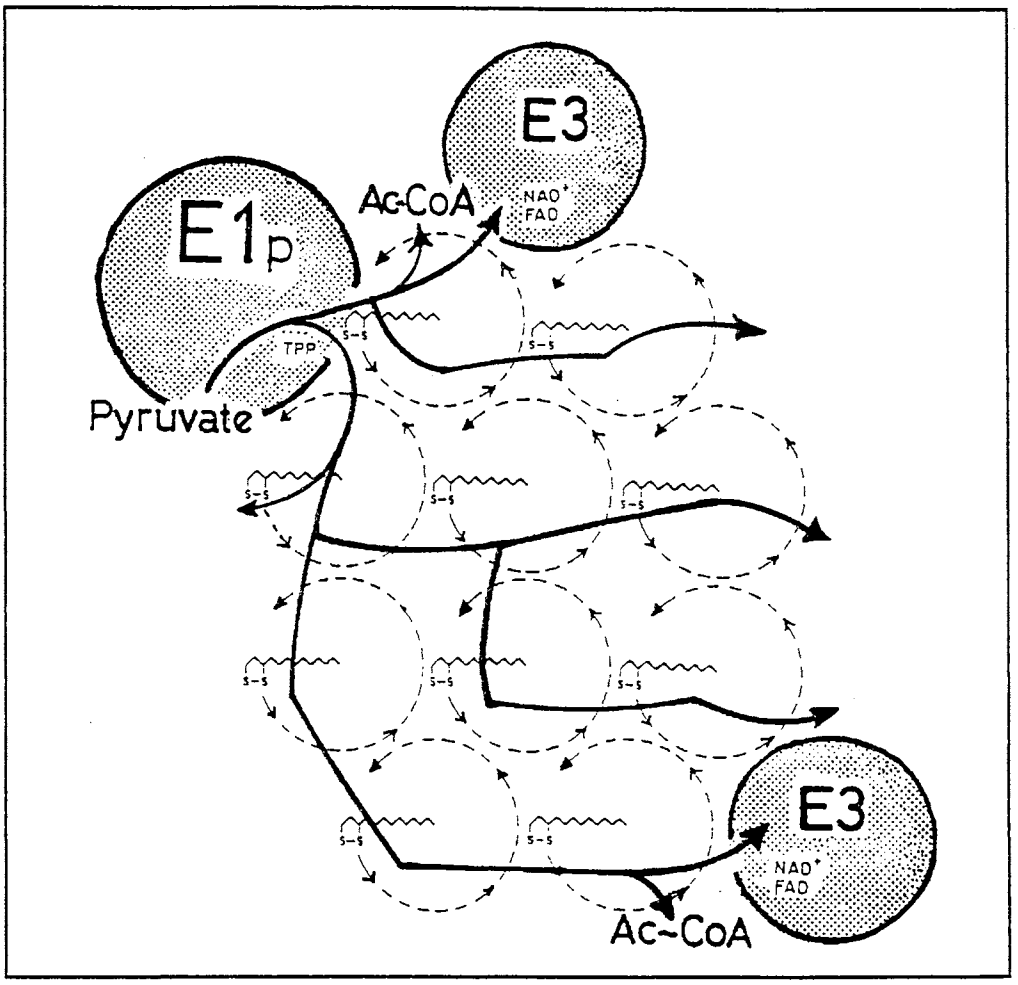


Figure I-5. Lipoyl Group Shuttle Network (16). Dashed line circles represent E2 subunits with lipoyl domains indicated by lipoic acid, the lysine attachment is not shown. Dark lines represent potential pathways for electron and acyl group transfer.

The organization of E2 subunits is similar across species in that the amino terminal region contains the lipoyl domains followed by the E3 binding domain and the dihydrolipoyl transacylase catalytic domain with the E1 binding domain at the carboxyl terminus. The number of lipoyl domains in each E2 varies with organism: *E. coli* α-keto glutarate DH has one, *E. coli* PDH has three, mammalian PDH has two and mammalian branched chain DH has one (22) (Fig. I-6).

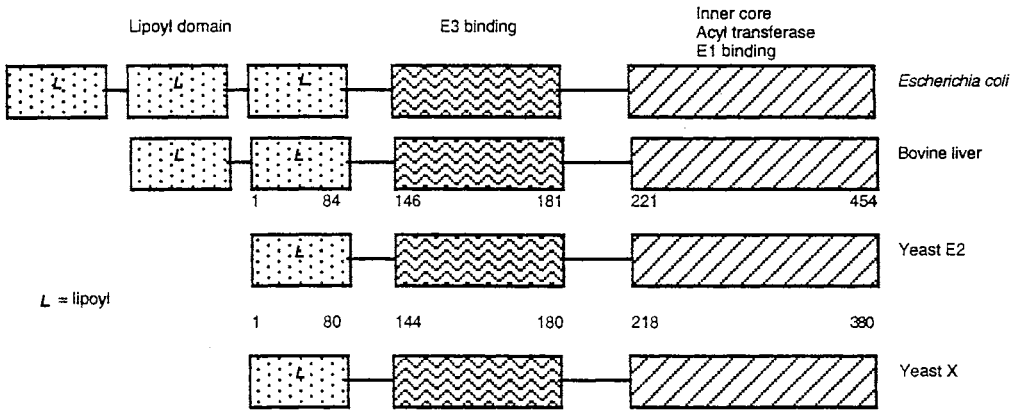


Figure I-6. Domain Structure and Lipoylation Pattern of Various E2 Subunits. All structures shown are of the indicated species PDH. Yeast X indicates the X or E3 binding protein.

Sequence analysis of 12 E2 subunit lipoyl domains reveals considerable conservation in the eleven amino acids around the lipoic acid attachment site as follows:

$$L_4\ E_9\ S_5\ D_9\ {}^{*}K_{12}\ A_{10}\ S_8\ M_6\ E_7\ V_6\ P_8$$

(Subscripts indicate the number of sequences in which the given amino acid is present and *K indicates the point of lipoic acid attachment.)

G. Activating System

Lipoic acid is covalently attached to the side chain of lysine residues in the enzymes in which it occurs. The lipoic acid is added to α-keto acid dehydrogenases as a post translational modification. In *Enterococcus faecalis* this is done by an enzyme system that requires ATP and Mg^{2+} and can be separated into two fractions, both are required for attachment (2). The reaction scheme in which AE1 activates lipoic acid by coupling it to AMP and then AE2 transfers the lipoyl moiety to the ε-amino group of lysine is shown in Fig. I-7.

AE1 + ATP + Lipoic Acid ⇔ AE1-lipoyl-AMP + PP

AE1-lipoyl-AMP + AE2 ⇔ Lipoyl-AE2 + AMP + AE1

Lipoyl-AE2 + apo-PDHC ⇒ Lipoyl-PDHC + AE2

Figure I-7. Reactions of the Lipoic Acid Activating System of *E. faecalis*. AE1 indicates activating enzyme 1 of the activating system and AE2 indicates activating enzyme 2 of the activating system, PP is pyrophosphate, apoPDHC is inactive pyruvate dehydrogenase complex without lipoic acid.

In *E. coli* all of the catalytic functions required for lipoic acid attachment are carried out by a single enzyme; however there are two pathways for lipoic acid ligation involving different enzymes. The enzyme produced by the *lplA* gene is responsible for the attachment of exogeneously supplied lipoic acid and the *lipB* gene product utilizes endogeneously synthesized lipoic acid (4, 8). *E. coli* lipoyl ligases are cross-species-active as the human inner lipoyl domain cloned into *E. coli* is properly lipoylated (23). Jordan and Cronan (13) showed that lipoic acid can also be attached by transfer directly from the acyl-carrier protein of the lipoic acid biosynthetic machinery to lipoic acid dependent enzymes without an ATP requirement in *E. coli* and mitochondria. These results provide the link between lipoic acid biosynthesis and lipoic acid ligation in *E. coli* and eucaryotic mitochondria.

Attachment of lipoic acid is similar in mammals to that of *E. faecalis* requiring two enzymes. Lipoate activating enzyme (24) generates lipoyl-AMP and then lipoyltransferase attaches lipoic acid to the lipoate-dependent enzymes. Fujiwara *et al.* (25) recently isolated two isoforms of bovine lipoyltransferase, LipTI and LipTII, and cloned and sequenced the LipTII cDNA. It contained a 1119 base pair reading frame encoding 373 amino acids with a 26 amino acid mitochondrial targeting sequence. The predicted amino acid sequence showed 35% identity to *E. coli* lipoate-protein ligase A (the *lplA* gene product).

H. Lipoamidase

1. Early Observations and *S. faecalis*

Seaman (26-28) treated crude tissues or cell extracts with adsorption alumina and observed a lowering of the lipoic acid content of the α-keto acid dehydrogenases. This effect required the presence of an enzyme which released protein bound lipoic acid. In 1958 Reed *et al.* (29) reported that an enzyme in *Streptococcus faecalis* inactivated *E. coli* PDH by removing bound lipoic acid (Fig. I-8) and that subsequent incubation with lipoic acid, ATP and a lipoic acid activating enzyme system restored PDH activity. This lipoic acid liberating enzyme was named lipoyl-X-hydrolase. An enzyme with similar hydrolase properties was reported from bakers' yeast (30). In 1963 Suzuki and Reed (31) reported on additional substrate specificity and devised a procedure for a 100-fold purification of lipoamidase from *S. faecalis.* The enzyme hydrolyzed methyl lipoate, lipoamide and ε-N-lipoyl-L-lysine and other lipoyl amino acids but not ε-N-biotinyl-L-lysine, ε-N-acetyl-L-lysine or ε-N-benzoyl-L-lysine. Based on the substrate specificities they called the enzyme lipoamidase instead of lipoyl-X-hydrolase. The enzyme has not been found in *E. coli* (Crookes Strain) (32, 33).

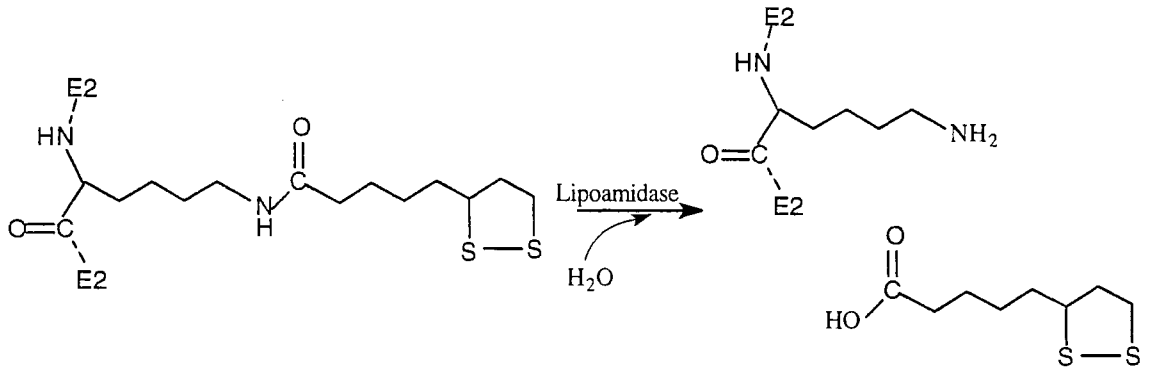


Figure. I-8. Lipoamidase Reaction. The structure on the left is lipoic acid attached to the ε-NH_2 of a lysine in the dihydrolipoyl transacetylase (E2) subunit.

2. Mammalian Lipoamidase and Relation to Biotinidase

No further work on lipoamidase was reported until 1987 when Oizumi and Hayakawa investigated mammalian lipoamidase. They devised an HPLC assay for lipoamidase based on the synthetic substrate lipoyl-*p*ABA (LA-*p*ABA) (34) and then examined various tissues from various organisms for lipoamidase. The assay detected a lipoamidase in human serum which has a pH optimum of 7 (35). A breast milk lipoamidase was purified 4,400-fold. The protein is a 135 kDa glycoprotein with a pI of 4.2, a pH optimum of 7, and has serine hydrolase properties (36). In various guinea pig tissues most lipoamidase activity occurs in a liver membrane fraction and the presumed protein has serine amidase properties. The active protein has a pI of 5.7 and an apparent molecular weight of 60 kDa by SDS-PAGE and 120 kDa by native PAGE (37). Human serum lipoamidase liberated lipoic acid from bovine PDH only after treatment with trypsin (38). It was speculated that the enzyme probably only functions in lipoic acid salvage. An enzyme from pig brain membrane fractions was purified by affinity chromatography using an Arg-Phe-NH_2 column (lipoyl based affinity columns were not effective) (39). The interaction of chelated iron with the pig brain lipoamidase was studied and it was found that chelated iron inhibits the enzyme while free iron does not (40).

Backman-Gullers *et al.* (41) compared human serum and breast milk lipoamidase using LA-*p*ABA and lipoyl-lysine as substrates. In serum LA-pABA and lipoyl-lysine cleaving activities responded differently to inhibitors suggesting different enzymes were responsible for the activity. They dubbed these lipoyl-lysine hydrolase (LLH) and lipoyl-*p*ABA hydrolase (LPH). LLH was activated by thiol compounds and EDTA and was inhibited by sulfhydryl-inhibitors while LPH was not activated by thiols or EDTA but was inhibited by sulfhydryl-inhibitors. In breast milk LA-*p*ABA and lipoyl-lysine cleaving activities were unaffected by any of the reagents that affect the serum activity. Serum LLH

and possibly LPH are cysteine hydrolases while no inference was made about milk lipoamidase. Using various tissues from rat and comparing lipoamidase and biotinidase activity Nilsson *et al.* concluded that liver LLH is definitely different from LPH and in fact LLH is biotinidase (42).

Other labs also noted inconsistencies in what was reported as lipoamidase. Specifically, Garganta *et al.* (43) concluded that pH profiles, molecular weight, thermostability profiles and inhibition by p-HMB and PMSF provided ample evidence that human serum lipoamidase is really biotinidase. Further evidence included the finding that a monospecific polyclonal antibody raised against biotinidase precipitated 95% of both lipoamidase and biotinidase activity and that children with profound biotinidase deficiency also had drastically reduced lipoamidase activity (43). Thus, most if not all, lipoamidase activity in serum is due to biotinidase.

Nilsson *et al.* (44) showed a concomitant deficiency in serum lipoamidase activity in a 21 month old boy with profound biotinidase deficiency using both the LA-*p*ABA and biotinyl-lysine assays. They concluded that lipoamidase and biotinidase are the same enzyme. However a small amount of residual activity was seen using the LA-*p*ABA assay. The investigators suggested that a small amount of modified biotinidase probably exists. In another study Nilsson *et al.* (45) showed that human serum lipoamidase and biotinidase activities copurified through 7 different purification steps. The resulting enzyme had a molecular weight by SDS-PAGE of 76 kDa and a pI of 4.

Hui *et al.* (46) cleaved the human milk lipoamidase with endoproteinase Lys-C and performed gas phase microsequencing on the resulting peptides. Sequence analysis showed this enzyme to be the bile salt-stimulated lipase (pancreatic cholesterol esterase EC # 3.1.1.13). They then cloned and expressed a recombinant rat pancreatic cholesterol esterase which showed both lipolytic and lipoamidase (LA-*p*ABA cleavage) activity. Mutagenesis of the recombinant gene by an amino acid substitution known to abolish esterase activity (His435→Gln435) resulted in loss of the lipolytic but not the lipoamidase

activity. The authors state that an enzyme in the digestive tract which is capable of both lipoylitic and lipoamidase activity must be of great importance in digestion and absorption of nutrients.

Further work on the pig brain enzyme by Oizumi *et al.* (47, 48) showed that >95% of the lipoamidase activity comes from membrane fractions. The enzyme was purified ~600 fold; it is a 140 kDa glycoprotein that hydrolyzes lipoyl-lysine but not biotinyl-lysine. It requires lipoic acid in the substrate but it hydrolyzes amide, ester or peptide bonds and thus has loose specificity as to the attachment site of lipoic acid. They classified this enzyme as a multiple hydrolase with an unknown function. Since the enzyme was membrane associated, they examined the effect of phospholipids and found that phosphatidylethanolamine induced a 2-fold increase in activity but that phosphatidylserine, cardiolipin, and phosphatidic acid induced an 80% reduction in activity (49). Subsequent studies showed that the pig brain enzyme releases membrane bound proteins which are glycosyl-phosphatidylinositol bonded and those which are myristolated but not integral membrane proteins. This activity was inhibited by lipoyl-lysine (50).

In summary, prokaryotic lipoamidase has been studied only briefly. It has been partially purified and found to cleave substrates with lipoic acid in an amide bond to a variety of other molecules including protein bound lysine but little else is known. Eucaryotic lipoamidases from many sources have been studied extensively and the proteins range in size from 60 kDa to 140 kDa. The substrate specificity ranges from simple LA-*p*ABA to the phosphatidylinositol tail of membrane associated proteins. Some enzymes with lipoamidase activity also have biotinidase or pancreatic cholesterol esterase activities.

I. Purpose of This Work

All of the enzymes concerned with the α-keto acid dehydrogenases, the attachment of lipoic acid to the ε-NH_2 of lysines, and the regulation of PDH complex activity have

been cloned and sequenced. Lipoamidase is the only enzyme that has not been cloned and sequenced.

Given the ambiguities about mammalian lipoamidases and the fact that *E. faecalis* lipoamidase has been partially purified, I chose the enzyme from *E. faecalis* as the starting point for further study of lipoamidase. The goal was to purifiy lipoamidase (from *E. faecalis*) either to homogeneity or to obtain sufficient purity so that a partial amino acid sequence could be determined. The partial amino acid sequence would make possible the construction of a nucleic acid probe to screen the existing *E. faecalis* 10C1 library.

The utility of lipoamidase in investigating the role of lipoyl moieties in various functions within the α-keto acid dehydrogenase complex has been established by Dr. Thomas Roche's group. Specifically, Stepp *et al.* (18) used lipoamidase and trypsin treatment in developing the lipoyl domain concept in E2 structure.

Potential uses of purified lipoamidase include study of:

1) role of lipoyl moieties in phosphorylation and dephosphorylation.
2) role of lipoyl moieties in proteins whose function is unknown.
3) role of lipoyl moieties in protein X function.
4) role of lipoyl moieties in the autoimmune disease, primary biliary cirrhosis.

The physiological function of lipoamidase has not been established. Is it a lipoic acid salvage enzyme? Does it have a role in the regulation of lipoyl-dependent enzymes? Or is it part of a multifunctional enzyme which includes one or both of these functions? There are many important questions that could be answered by an intensive study of lipoamidase.

CHAPTER II

MATERIALS AND METHODS

A. Materials

All chemicals and materials were from Sigma unless specifically indicated otherwise. Skim milk and tomato juice were from a local grocery store. Yeast extract and tryptone were from Difco. Casein was from United States Biochemical. Salts in the 'Salts B' solution were from J.T. Baker.

B. Growth of Lipoic Acid Deficient Enterococcus faecalis

Enterococcus faecalis (E. faecalis) was obtained from American Type Culture Collection, (ATCC #11700, strain 10C1). The dehydrated culture was rehydrated in 0.4 ml of ATCC medium #17 (Table 1) and then transferred to 6 ml of the same medium and incubated at 37 °C for 24 h with shaking. Working stock cultures were kept as nutrient agar deep stabs (Table 1) which were made by inoculating with two stabs from the ATCC medium #17 cultures and incubated at 37 °C until growth was evident along the stab lines. Two stock tubes were kept at 4 °C for one month; one was kept to inoculate the next set of stock tubes and the other was used to inoculate cultures for protein purification. A glycerol-enriched stock culture was also generated by growing an overnight 37 °C culture

in nutrient broth (5 ml final volume). After incubation, glycerol was added to 15% final concentration and 0.5 ml aliquots were stored at -80 °C in screw top microfuge tubes.

Table 1. Growth Media Formulations

ATCC #17

Ingredient	Amount added per liter
Skim milk	100 g
Tomato juice	100 ml
Yeast extract	5.0 g

Tomato juice was filtered through #1 Whatman paper and left overnight at 4 °C then titrated to pH 7.0 before use.

PYTG Medium for *E. faecalis*

Ingredient	g per liter	Final Concentration (%)
K_2HPO_4	5.0	0.5
Yeast extract	10.0	1.0
Tryptone	10.0	1.0
*Glucose	1.0	0.1

pH 7.0, *Glucose is autoclaved separately, then added to the medium at time of inoculation. Agar tubes contained 2% w/v agar.

Lipoic Acid Deficient Medium[1]

Ingredient	Amount added per liter final
[2]Acid hydrolyzed casein	200 ml (10.0 g)
[2]Enzyme hydrolyzed casein	200 ml (7.5 g)
[3]Glucose	3.0 g
K_2HPO_4	5.0 g
Sodium thioglycolate	100 mg
DL-tryptophan	200 mg
L-cysteine	200 mg
Adenine	25 mg
Guanine	25 mg
Uracil	25 mg
Nicotinic acid	5 mg
Riboflavin	1 mg
Pyridoxine•HCl	1 mg
Thiamine•HCl	1 mg
Calcium pantothenate	1 mg
Folic acid	10 µg
Biotin	1 µg
[4]Salts B	5 ml

[1] (51)

[2]Commercial acid and enzyme hydrolyzed casein (United States Biochemical) were treated as follows: 222.2 g of acid hydrolyzed and 167 g of enzymatic hydrolyzed casein were dissolved separately in 4 liter each of water. Then 50 g of activated charcoal was added to each and stirred for 30 min. The solutions were filtered through Whatman #1 paper and 100 ml of glacial acetic acid and 50 g more activated charcoal were added. The solutions were stirred for 30 min, filtered, diluted to 4.5 liters and stored at 4 °C.

[3]Put glucose into solution and autoclave separately

[4]Salts B (29): 10 g $MgSO_4•7H_2O$, 0.5 g NaCl, 0.5 g $FeSO_4•7H_2O$, and 0.38 g $MnSO_4•H_2O$ in 250 ml final volume.

C. *Protein Concentration Determination*

1. Pierce BCA Assay

This assay was done as per manufacturer's instructions using BSA as a standard. On occasion the assay was performed using a microwave oven as incubation heat source with a 100 ml beaker of water included as a heat sink as per Aikins and Taun (52).

2. Bradford Assay

This assay was used as per Bollag and Edelstein (53) with BSA as a standard.

3. Spectrophotometric Assay

This assay was done as per Kalb and Bernlohr (54). The spectrophotometer was zeroed with sample buffer. Absorbance values were determined with 2 or 3 different dilutions. Protein concentration was determined using the empirical formula

$$\mu g/ml = (183 \times A_{230}) - (75.8 \times A_{260})$$

for samples with an A_{230} less than 1.5.

D. *Sample Concentration*

1. Ultrafiltration

To concentrate protein prior to column chromatography the method of choice was usually ultrafiltration using a device such as Micro-con®, Centri-con® or Centri-prep® from Amicon. Before use, these were treated with 10% polyethyleneglycol (PEG) as recommended by the manufacturer to reduce protein loss on the membrane. This method was also used to desalt samples or change the buffer.

The protein solution was put into the device and centrifuged as per manufacturer's instructions. For desalting or buffer exchange the solution was diluted with new buffer and centrifuged 4-5 times. The resulting solution was recovered as per manufacturer's instructions.

2. TCA precipitation

To concentrate protein samples for SDS-PAGE, 100% (w/v) TCA was added to a final concentration of 10% TCA. The mixture was vortexed for 10 s, set on ice for 30 min, and the resulting precipitate was collected by centrifugation at 1000 × g for 5 min. The supernatant solution was poured off, the pellets washed in acetone and then air dried. The dried pellets were dissolved in a minimal amount of SDS sample buffer and boiled prior to electrophoresis (55).

3. Phenol/Ether precipitation

TCA precipitation often leaves an acidified pellet unsuitable for SDS-PAGE. To overcome this, phenol/ether precipitation was performed as described by Sauve *et al.* (56). An equal volume of water-saturated phenol was added to dilute protein samples which were vortexed for 20 s and centrifuged at 12,000 × g for 5 min. The upper phase was discarded and the lower phase was extracted twice with 2 volumes of ether. The resulting aqueous phase was dried in a Speed Vac. The dried samples were redissolved in SDS-PAGE sample buffer and boiled prior to electrophoresis.

4. Speed Vac

If the volume reduction required was small and salt concentration was not a concern, samples were concentrated in a Speed Vac as per manufacturer's instructions, being careful to not process to dryness unless desired.

E. Assays

The natural substrate of lipoamidase is lipoic acid attached to the ε-amino group of lysine residues of proteins; lipoamidase can also cleave small synthetic molecules containing lipoic acid in an amide or ester linkage. Three lipoamidase assays were used:

1) a PDH based assay (2) which measures lipoamidase activity by determining the decrease in PDH activity due to lipoic acid removal. The assay will be referred to as the PDH inactivation assay.
2) a LA- *p*ABA hydrolysis assay (57) which measures *p*ABA released.
3) a lipoyl domain inactivation assay which measures the release of lipoic acid from a human lipoyl domain peptide. Lipoamidase reduces the ability of a recombinant lipoyl domain to participate in a coupled oxidation reduction reaction with exogenous E3 subunit of the α-keto acid dehydrogenases (dihydrolipoyl dehydrogenase) and DTNB (58). The assay will be referred to as the LipDom inactivation assay.

F. PDH Assay

1. Principle

The PDH assay used is a modified version of the dismutation assay originally described by Korkes *et al.* (59); it is modified as described by Reed *et al.* (2). In this assay acetyl-CoA generated by PDH from pyruvate is converted to acetyl-phosphate by phosphotransacetylase. The acetyl-phosphate is then non-enzymatically converted to an acetyl-hydroxamic-Fe complex which is quantitated by measuring absorbance at 540 nm. Lactate dehydrogenase is included to regenerate NAD^+ (Fig. II-1). One unit of PDH activity generates 1 μmole of acetyl-phosphate per h at 37 °C.

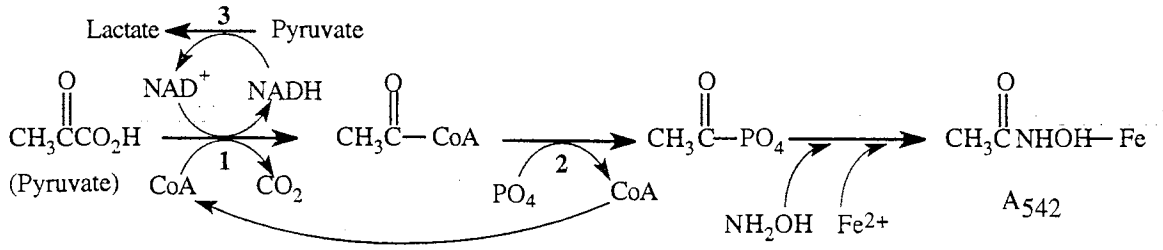


Figure II-1. PDH Assay Reaction Scheme. Reaction 1 is catalyzed by lipoylated PDH (holoPDH), reaction 2 is catalyzed by phosphotransacetylase (PTA), reaction 3 is catalyzed by lactate dehydrogenase (LDH).

The generation of acetyl-CoA depends on the presence of all PDH cofactors including protein bound lipoic acid. Using a two stage approach, the assay described can determine the activity of enzymes which attach lipoic acid to the E2 with a resulting increase in PDH activity or determine the activity of enzymes which remove lipoic acid with a resulting decrease in PDH activity.

To assay for lipoic acid attachment by the lipoic acid activating (lipoyl ligase) system the first stage of the assay utilizes apoPDH, lipoic acid and ATP as substrates. The decarboxylation cofactor TPP is also added in stage one to insure it is bound to PDH E1. In the second stage pyruvate is added as substrate for the newly activated PDH and phosphate is added as substrate for PTA. NAD^+ and CoA are added as a cofactors. CoA is regenerated by reaction 2 and NAD^+ is regenerated by reaction 3 (Fig. II-1) (even though amounts are added to assure saturating quantity). This is referred to as the apoPDH activation assay.

To assay for lipoic acid removal by lipoamidase the first stage of the assay utilizes holoPDH as substrate. Pyruvate and CoA and NAD^+ are added in the second stage as substrates for any active PDH remaining. This is referred to as the PDH inactivation assay.

2. Reagents for apoPDH Activation Assay

Stage One

0.01 ml of 1.0 mg/ml DL-lipoic acid (0.005 µmole)
0.01 ml of 0.96 mg/ml TPP (0.02 µmole)
0.01 ml of 1.0 mg/ml ATP (0.02 µmole)
0.01 ml of 9.6 mg/ml $MgSO_4$ (0.8 µmole)
0.006 ml of 1.0 M potassium phosphate buffer, pH 7.0 (6.0 µmole)
0.004 ml of 12.0 mg/ml cysteine (1.0 µmole) [fresh daily]
apoPDH (*E. faecalis*)
an activating system preparation
H_2O to 0.25 ml final volume

Stage Two

0.25 ml complete stage one reaction mixture
0.1 ml of 1.0 M potassium phosphate buffer, pH 7.0 (100 µmole)
0.8 unit phosphotransacetylase (PTA) (Sigma P-2669)
9.6 units of lactate dehydrogenase (LDH) (Sigma L-2881)
0.1 ml of supplement containing CoA (0.1 µmole), NAD^+ (0.23 µmole) and cysteine (6.4 µmole) [fresh daily]
H_2O to 1.0 ml (with 0.05 ml of pyruvate)
The reaction is initiated with the addition of 0.05 ml of 1.0 M potassium pyruvate (50 µmole)

Color Development Reagents

1.0 ml of 0.1 M potassium citrate buffer, pH 5.4
1.0 ml of pH 6.2 NH_2OH (4.0 M NH_2OH brought to pH 6.2 by addition of 3.5 M NaOH) [fresh daily]
3.0 ml of Hoagland reagent (prepared by adding 30.0 ml of concentrated HCl, 50 g of $FeCl_3$ and 25.0 g of trichloroacetic acid to 470 ml of H_2O).

3. Procedure for the apoPDH Activation Assay

Stage one is initiated with the addition of apoPDH and the mixture is vortexed and incubated at 30 °C for various time depending on the activating system preparation. At the end of the stage one incubation the stage two components are added, the mixtures vortexed and incubated at 30 °C for 30 min.

The colored product is developed by pipetting 0.950 ml of the stage two mixture into a 13 X 100 mm test tube containing 1.0 ml of 0.1 M potassium citrate buffer pH 5.4

and 1.0 ml of fresh pH 6.2 NH_2OH. The tubes are vortexed and incubated at room temperature for 10 min. Then 3.0 ml of Hoagland reagent is added and the tubes are vortexed and then centrifuged at 1000 × g for 5 min to remove precipitated protein. The absorbance of the supernatant solution is then determined at 540 nm against a blank containing all components except the activating system. The amount of acetyl phosphate is determined by calculation based on a standard curve of succinic anhydride (60) (Fig. II-2). Controls typically include apoPDH without activating system and activating system without apoPDH.

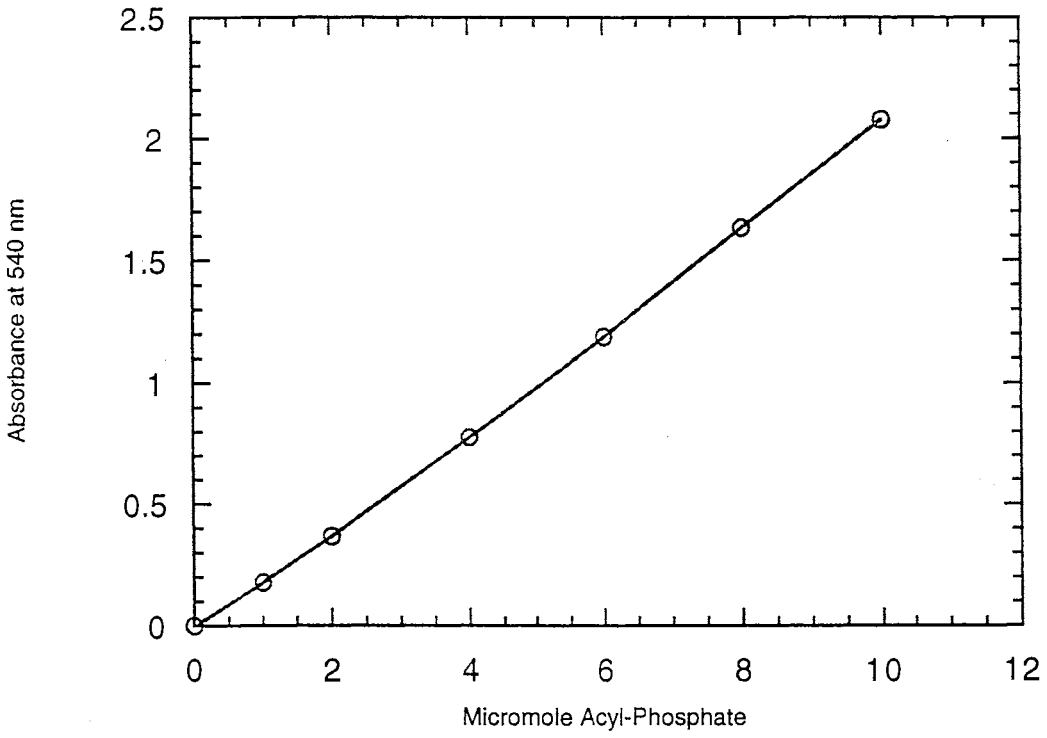


Figure II-2. Standard Curve for Acyl Phosphate. This curve was generated with succinic anhydride (60) in 0.02 M potassium phosphate buffer, pH 7.0 in 0.95 ml total volume then treated as described in the color development procedure. Succinic anhydride yields 80% of the absorbance of acyl phosphates. This curve has been corrected by 20% from the succinic anhydride values as per (60). The equation for the linear regression line determined from the data is: $y = -0.031 + 0.208x$. $R = 1$.

4. Reagents for the PDH Inactivation Assay

Stage One

0.006 ml of 1.0 M potassium phosphate buffer, pH 7.0 (6.0 µmole)
3-5 units of holoPDH (one unit = 1 µmole of acyl-phosphate / hour of incubation).
up to 0.094 ml of a lipoamidase preparation
H_2O to 0.20 ml final volume

Stage Two

0.20 ml of stage one reaction
0.01 ml of 0.96 mg/ml TPP (0.02 µmole)
0.01 ml of 9.6 mg/ml $MgSO_4$ (0.8 µmole)
0.004 ml of 12.0 mg/ml cysteine (1.0 µmole) [fresh daily]
0.1 ml of 1.0 M potassium phosphate buffer, pH 7.0 (100 µmole)
0.8 unit Sigma PTA
9.6 units of Sigma LDH
0.10 ml of supplement containing CoA (0.1 µmole), NAD^+ (0.23 µmole) and cysteine (6.4 µmole) [fresh daily]
H_2O to 0.95 ml (with 0.05 ml of pyruvate)
The reaction is initiated with 0.05 ml of 1.0 M potassium pyruvate (50 µmole)

5. Procedure for PDH Inactivation (lipoamidase assay)

Stage one is initiated with the addition of holoPDH and the mixture is vortexed and incubated at 30 °C for various times depending on the lipoamidase preparation (1-3 hours). At the end of stage one the stage two components are added and the mixtures are vortexed and incubated at 30 °C for 30 min. Color development is identical to that described in the procedure for apoPDH activation. Controls typically include holoPDH alone and lipoamidase alone.

G. Pyruvate Dehydrogenase Inactivation / Reactivation Assay

The PDH inactivation assay demonstrates a "loss of function" which may have many causes including, but not limited to, regulation of PDH by product inhibition, cleavage of the hinge region of E2 subunit or other locations, disruption of PDH subunit association, inhibition of assay accessory enzymes PTA or LDH, and utilization of assay

components by other enzymes. Therefore the only way to be certain that an observed loss of PDH activity is due to removal of lipoic acid by lipoamidase is to reattach lipoic acid and observe an increase in PDH activity or reactivation.

Two methods are used to separate PDH from lipoamidase before reactivation. The sample is either centrifuged at 144,000 × g for 1 h to pellet the PDH or filtered through a 100,000 MW cutoff centrifugal ultrafiltration device which retains PDH but not lipoamidase.

For each lipoamidase fraction to be assayed 3-5 units of holoPDH in 0.1 ml; 0.05 ml lipoamidase fraction; 0.05 ml of KPO_4 buffer, pH 7; and 0.05 ml H_2O are combined in a 1.5 ml microfuge tube, mixed and incubated at 30 °C for 1 h. A control containing an additional 0.05 ml of H_2O in the place of the lipoamidase fraction is also included. At the end of 1 h incubation the PDH is separated from the lipoamidase by either ultracentrifugation or ultrafiltration.

When ultracentrifugation is used, each 0.25 ml sample is centrifuged at 144,000 × g for one h. The pellet is then dissolved in 0.11 ml of KPO_4 buffer pH 7, two aliquots of 0.05 ml each are put into 1.5 ml microfuge tubes and the stage one reagents are added. To one tube 0.1 ml of activating system is added and to the other 0.1 ml H_2O is added. These are incubated at 30 °C for 30 min at which time the stage two reagents are added and the PDH assay is completed as previously described.

When ultrafiltration is used, each 0.25 ml sample is placed into a passivated Micro-Con® 100 and centrifuged until all of the solution that can has passed through the filter, the concentrate is then recovered and diluted to 0.11 ml. Two aliquots of 0.05 ml are removed and placed into 1.5 ml microfuge tubes and treated as described for 144,000 × g treatment. Micro-Con® devices are passivated by soaking in 5% polyethylene glycol for 30 min and then washing with H_2O before use.

Controls include holoPDH without lipoamidase as a negative control and activating system plus apoPDH as a positive control for the activating system.

H. LA-pABA Hydrolysis Assay

1. Synthesis of LA-*p*ABA

LA-*p*ABA was synthesized from DL-lipoic acid and Na-*p*ABA (sodium para-amino benzoic acid) as per (43). Lipoic acid (5.0 mmole, 1.1 g) and triethylamine (5.0 mmole, 0.51 g) were dissolved in 10.0 ml of THF and chilled in an ice/salt bath. Isobutylchloroformate (5.0 mmole, 0.68 g) was added dropwise with stirring. After 10 min stirring, *p*-ABA (5.0 mmole, 0.76 g dissolved in 5.0 ml of 1.0 M NaOH) was added and the mixture stirred overnight at room temperature. The solution was then extracted twice with 40.0 ml of benzene to remove unreacted *p*-ABA. The slightly yellow upper layer was discarded and the deeply yellow lower phase was diluted to 50.0 ml with H_2O. Then 12.0 ml of 0.5 M HCl was added to precipitate LA-*p*ABA. The resulting milky yellow mixture was filtered through Whatman #1 filter paper in a Büchner funnel. The pale yellow precipitate left on the filter was washed with a mixture of ethanol:H_2O:glacial acetic acid (45:45:10) to remove unreacted *p*ABA. The washings were repeated until the filtrate was relatively free of *p*ABA as determined by the Bratton-Marshall reaction[1] (61). Washing was done with 15 or 30 ml at a time for a total of 800 ml.

After determining the filtrate was relatively free of *p*ABA, the yellow LA-*p*ABA precipitate was then washed with H_2O until the pH was ~7.0. This took about 2,500 ml. The compound was then dried over desiccant for ~ 60 h. yielding 1.13 g of dry material (~65%yield).

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[1] The Bratton-Marshall reaction was performed by adding 1.0 ml of 0.1% Na nitrite to 10.0 ml of the filtrate, mixing, and incubating 3 min at room temperature, then 1.0 ml of 0.5% ammonium sulfamate was added and the solution was mixed and incubated 3 min at room temperature, then 1.0 ml of 0.1% NEDD (N-1-napthylethylenediamine) was added and the solution was mixed and incubated 3 min at room temperature. The resulting *p*ABA-NEDD conjugate was quantitated by measuring absorbance at 550 nm.

2. Assay Principle

Lipoamidase cleaves LA-*p*ABA to yield lipoic acid and *p*ABA (62), *p*ABA is then quantitated by coupling to NEDD by the Bratton-Marshall reaction (61) and measuring absorbance at 550 nm against a reagent blank (Fig. II-4).

3. Assay Procedure

Reagents

up to 0.5 ml of enzyme fraction
0.25 ml of 20 mM potassium phosphate buffer pH 7.0
0.25 ml of 600 μM LA-*p*ABA
H_2O to 1.0 ml as required

The assay for lipoamidase is performed as per Garganta and Wolfe (43) by combining enzyme fraction with buffer and water, initiating the reaction with LA-*p*ABA, and incubating tubes at 30 or 37 °C for at least one h. At the end of the incubation the reaction is stopped by acidification with 0.10 ml of 100% TCA. Precipitated protein is removed by centrifugation at 2000 × g for 10 min. An aliquot of the supernatant solution (typically 0.95 ml) is removed to a 10 × 75 mm test tube and 0.1 ml of 0.1% Na nitrite is added. This is mixed well and incubated at room temperature for 3 min and then 0.1 ml of 0.5% ammonium sulfamate is added and the tubes are mixed and incubated at room temperature for 3 min. Then 0.1 ml of 0.1% NEDD is added, the solution is mixed and incubated at room temperature for 3 min, then vortexed for at least 10 s to remove gas bubbles which interfere with absorbance measurements. Each tube is then measured at 540 nm against a reagent blank. Controls include reagents only, *p*ABA alone as a positive control, LA-*p*ABA alone and sometimes the enzyme fraction of interest alone. The amount of *p*ABA released from LA-*p*ABA is determined from the equation generated from a standard curve for *p*ABA as described (Fig. II-3). The assay scheme is diagrammed in Fig. II-4.

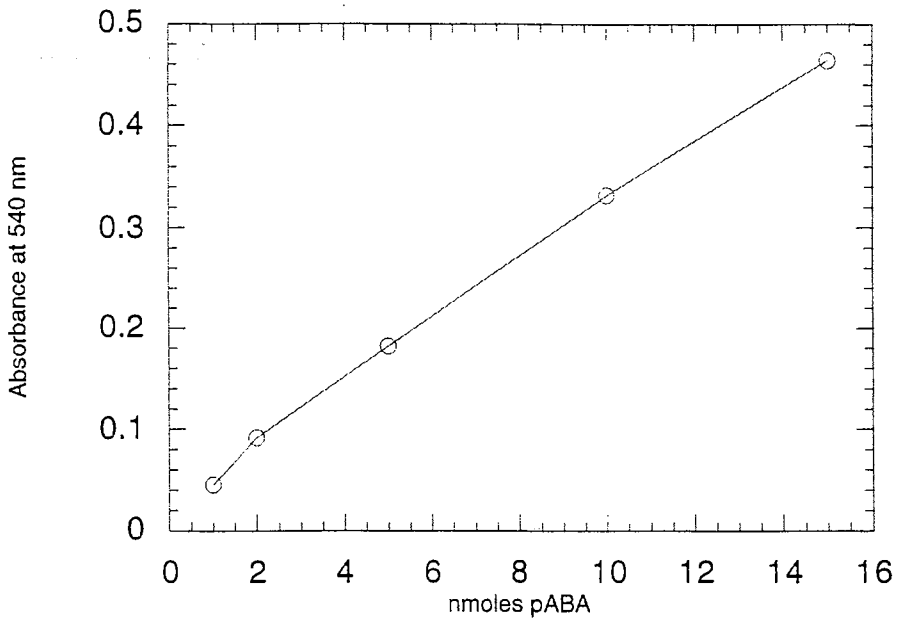

Figure II-3. Standard Curve for *p*ABA. Aliquots of *p*ABA were added to 0.25 ml of 20 mM potassium phosphate buffer, pH 7.0 and H_2O to 1.0 ml final volume and then color developed as described. Linear regression analysis gives the equation y = 0.030x +0.018 with a regression coefficient of R = 0.999.

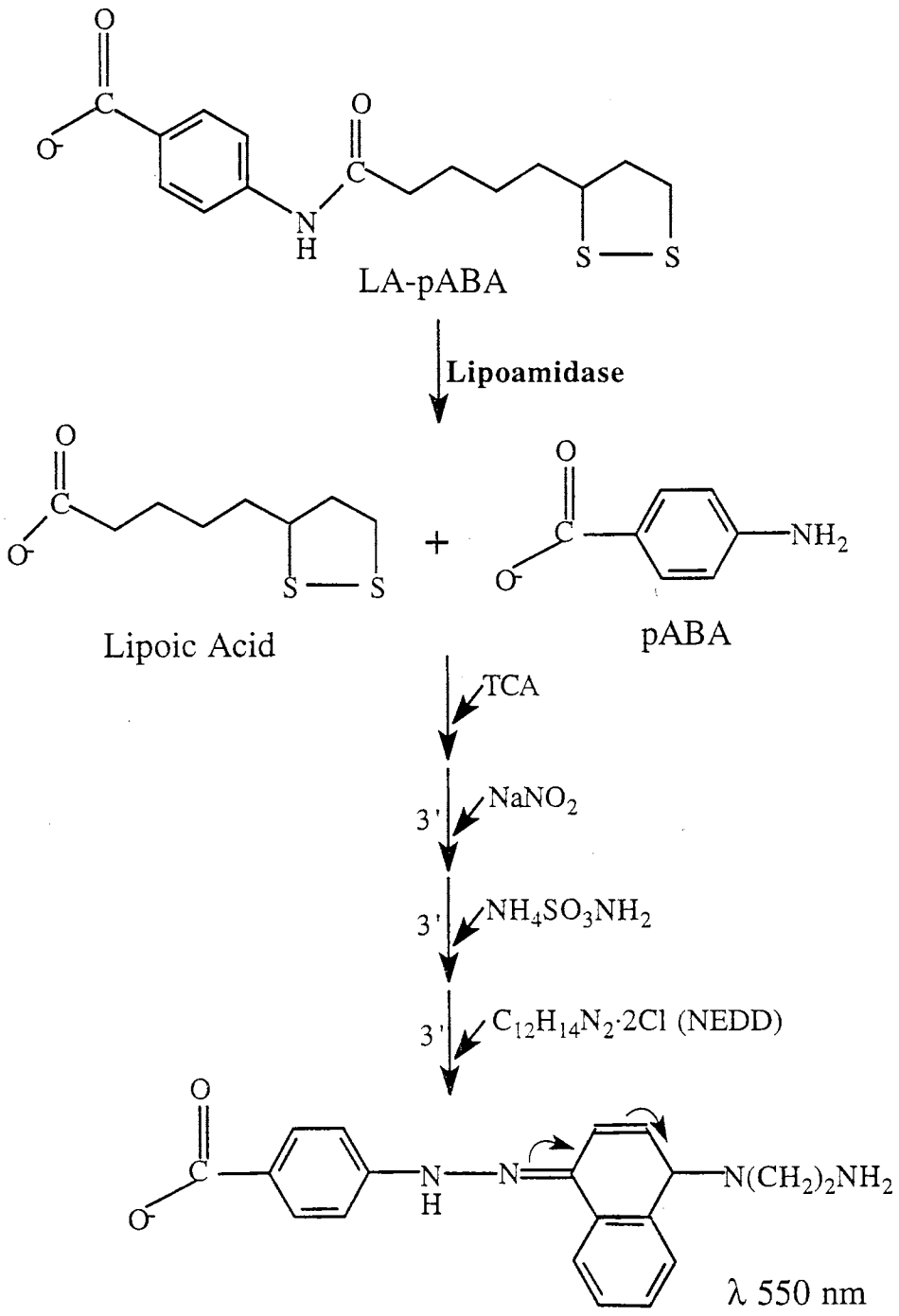

Figure II-4. LA-*p*ABA Cleavage Assay Scheme. LA-*p*ABA is cleaved by lipoamidase to yield lipoic acid and *p*ABA. *p*ABA is conjugated to NEDD through consecutive addition of TCA, sodium nitrite, ammonium sulfamate and NEDD. The *p*ABA -NEDD conjugate is determined at 550 and quantitated using a *p*ABA standard curve.

I. E3/DTNB Recycling Assay (LipDom Assay)

1. Principle

The procedures for this assay and *E. coli* carrying the pGEX-2T plasmid with the GST-lipoyl domain fusion protein were kindly provided by Dr. Thomas Roche, Kansas State University. The lipoyl domain used consists of amino acids 1-98 of the human PDH E2 subunit fused to glutathione S-transferase (GST, Pharmacia). It is supplied in a pGEX-2T plasmid harbored and produced in *E. coli* host strain JM-109. The growth, purification procedures and assay procedures are essentially as described by Liu *et al.* (63). The procedure measures the reaction of dihydrolipoyl moieties with DTNB to generate colored thionitrobenzoate (TNB) in a time course assay. The sensitivity of the assay is enhanced by recycling lipoyl domains by E3 (Fig. II-5). Since lipoyl domain activity is dependent on the presence of bound lipoic acid and each lipoyl domain contains only one lipoic acid this assay can measure lipoamidase activity as a decrease in TNB production. Neither free lipoic acid nor lipoyl-lysine are as efficiently recycled by E3 as complete lipoyl domain (64) (Fig. II-6). However lipoyl-lysine does have ~25 % the activity of lipoyl domain.

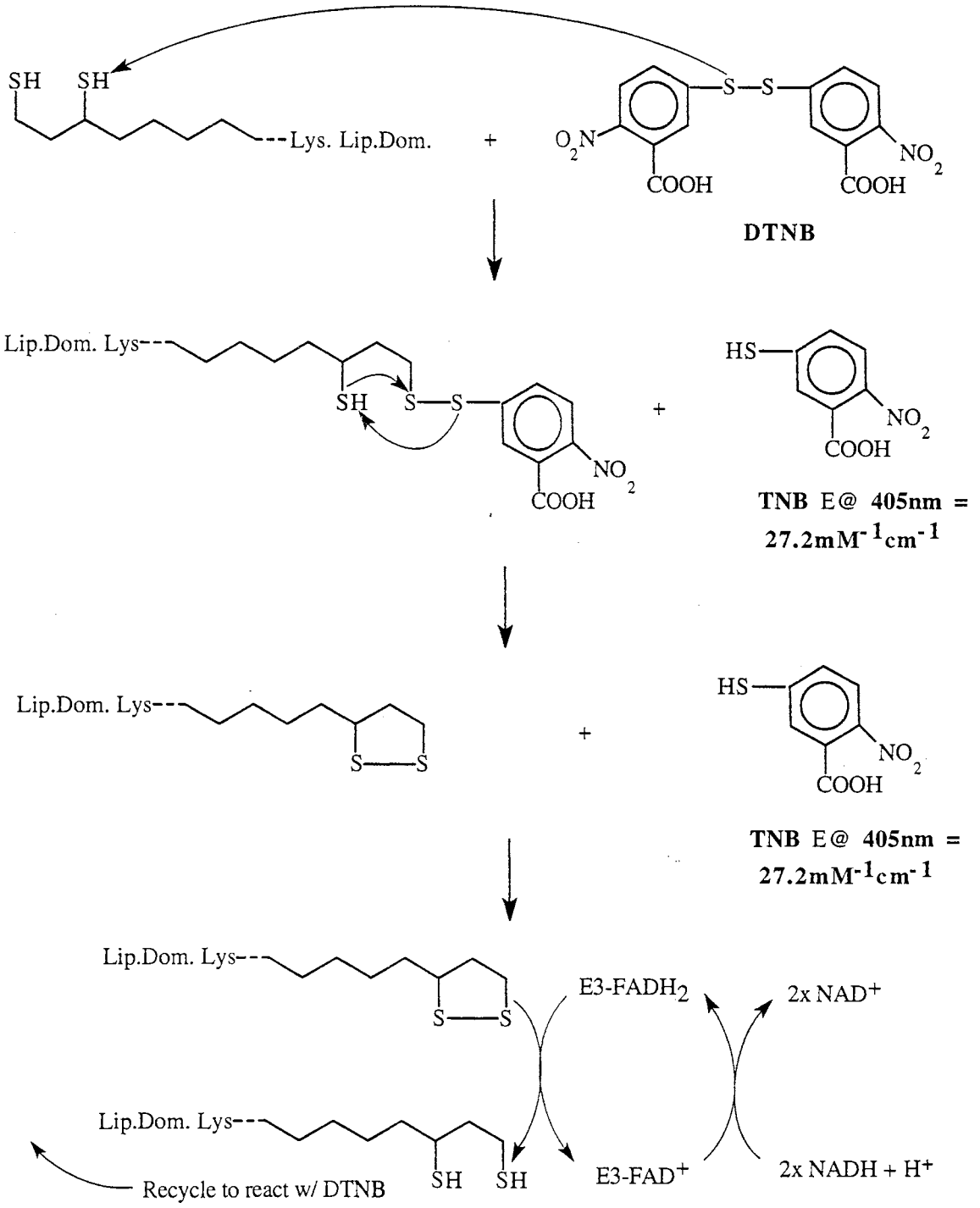

Figure II-5. Reaction Scheme E3/DTNB Recycle Assay (LipDom inactivation). Reduced lipoyl domain is oxidized by DTNB with the generation of 2 molecules of TNB. The oxidized lipoyl domain is then reduced by the $FADH_2$ associated with the PDH E3 subunit and recycled for further reaction with DTNB. The E3 FAD^+ is reduced by solution NADH completing one cycle.

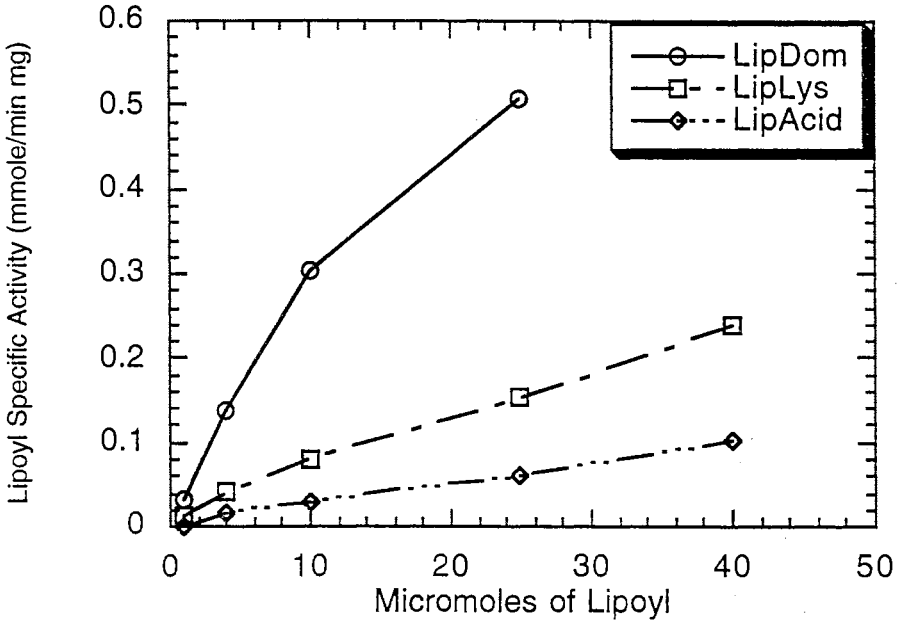

Figure II-6. Lipoyl Specific Activity in the E3/DTNB Recycling Assay with Different Lipoyl Moieties. The assay was performed as described by initiating the reaction with the indicated amounts of lipoyl moiety. LipDom is lipoylated recombinant amino acid 1-98 of human E2 fused to glutathione S-transferase, LipLys is lipoic acid linked to the ε-NH_2 of lysine, LipAcid is free lipoic acid. Data points are the average of three replicates of TNB production measured over 1 min.

2. Lipoyl Domain Production

E. coli harboring the lipoyl domain plasmid was grown to an OD_{600} of 0.7-0.8 at 37 °C in LB medium containing 0.01% glucose, 1 mM ampicillin and 0.2 mM lipoic acid and then induced to express the fusion protein by the addition of IPTG to 1 mM. The cells were incubated for an additional 5.5 h and then harvested by centrifugation (10,000 × g at 4 °C for 10 min). The cell pellet was washed twice by resuspension in 50 mM Tris buffer pH 8, containing 150 mM NaCl and 10 mM EDTA and pelleted at 10,000 × g. The pellet was weighed and then suspended in phosphate buffered saline, (PBS) pH 7.2 containing 1% Triton X-100; 5 mM EDTA; 1 μg/ml leupeptin; 1 μg/ml aprotinin; and 0.1 mM PMSF at 10 ml of buffer per g cells. Lysozyme was added at 10 mg per g cell paste and the

mixture was stirred on ice for 30 min. The cell mixture was then sonicated at ~50% full power with a Heat Systems Ultrasonics instrument for 10 × 1 min cycles with 1 min cooling in between. Cellular debris was pelleted and the lysate clarified by centrifugation at 10,000 × g at 4 °C for 5 min. GST activity of the fusion protein was determined by monitoring the formation of DNB-S-GSH from 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) as an increase in absorbance at 340 nm.

The reaction mixture contained 0.890 - 0.899 ml of 0.1 M $NaPO_4$, 50 µl of 20 mM glutathione (GSH) and 50 µl of 20 mM CDNB in a 1 ml final volume (including GST-LipDom). After mixing and measuring baseline absorbance at 340 nm, the reaction was initiated by adding 1-10 µl of the GST-LipDom preparation and monitoring A_{340}. The total mg of GST was calculated using the molar extinction coefficient of DNB-S-GSH of 9.6 $mM^{-1}cm^{-1}$ to convert ΔA_{340} to Δ mmole. Assuming 1.0 mg of GST will produce DNB-S-GSH at a rate of 0.014 mmole/min, the mg of GST can be calculated and the mg of lipoyl domain can be calculated based on a ratio of 3 mg GST:1 mg lipoyl domain.

GST activity

$$\frac{\Delta A}{\text{min}} \times \frac{\text{mmole}\cdot\text{cm}}{9.6\, L_A} \times \frac{1}{\text{cm}} \times \frac{L_\Delta}{L_E} = \frac{\text{mmole}}{L_E\cdot\text{min}}$$

GST total activity

$$\frac{\text{mmole}}{L_E\cdot\text{min}} \times \text{total } L_E = \frac{\text{mmole}}{\text{min}} \quad \text{---->} \quad \frac{\text{mmole}}{\text{min}} = \text{TA}$$

mg GST

$$\frac{1 \text{ mg GST}}{.014 \text{ TA}} \times \text{TA} = \text{mg GST}$$

Figure II-7. Calculation of mg GST for Determination of Lipoyl Domain Produced. L_E is volume of enzyme added to assay, L_A is total volume of assay mix. TA is GST Total Activity.

The lysate was then combined with GSH-Sepharose 4B gel (5 mg of GST per ml of gel) which had been equilibrated with 10 bed volumes of PBS, pH 7.2 containing 1.0% Triton X-100. The gel-protein mixture was shaken at room temperature for 30 min, centrifuged at 1,000 × g and the supernatant solution carefully decanted and assayed for GST activity. If activity was present, the supernatant solution was saved at -80 °C and the

remaining GST-LipDom purified later. The gel was then washed 8 times with 2 volumes of PBS, pH 7.2 containing 1.0% Triton X-100 followed by 3 washes of 2 volumes of 50 mM Tris, pH 8.0 containing 150 mM NaCl. The Tris washes were continued until A_{280} was below 0.001. The gel was then transferred to a glass column, allowed to settle and the buffer drained down to the top of the column. At this point 3 volumes of 50 mM Tris pH 8.0 containing 150 mM NaCl and 5 mM GSH were carefully added to the top of the column and any disturbed gel allowed to settle. The column was then eluted at ~1 ml/min with monitoring at 280 nm. The protein peak was collected and then placed into Centri-Prep® 30 ultrafiltration devices that had been pre-treated with PEG. The buffer was changed to 20 mM potassium phosphate buffer pH 7.0 containing 0.04 mg/ml EGTA, 0.01 µg/µl leupeptin and 0.01 µg/µl aprotinin by repeated processing in the Centri-Prep® 30 and dilution in the new buffer. The solution was brought to protein concentration of ~15 mg/ml. The column was regenerated by washing with 3 column volumes of 50 mM Tris pH 8.0 containing 150 mM NaCl and 5 mM GSH, followed by 3 column volumes of PBS, pH 7.2 containing 2.85 M NaCl and 1 mM EDTA. The gel was stored in 10% ethanol at 4 °C. The concentrated GST-LipDom preparation was distributed in 0.1 ml aliquots and stored at -80 °C and hereafter referred to simply as LipDom.

3. E3/DTNB Assay Procedure

Reaction Mix

50 µl of 0.2 M sodium phosphate buffer, pH 7.4, containing 2 mM EDTA
20 µl of aqueous solution of 3 mM NADH and 1 mM NAD^+
20 µl of 1.5 mM DTNB in 50 mM sodium phosphate buffer, pH 7.4
0.1- 0.5 µg of E3 (Lipoamide dehydrogenase Sigma L-2002)
50 - 90 µg GST-LipDom (10-40 µM final concentration)
H_2O to a final volume of 0.2 ml (including the GST-LipDom).

The spectrophotometer is zeroed with the reaction mix without GST-LipDom at 405 nm. The reaction is initiated by the addition of lipoyl domain and the progress of TNB

formation followed for at least 45 s. The molar extinction coefficient for TNB is 27.2 $mM^{-1}cm^{-1}$.

To measure lipoamidase activity, ~225 µg lipoyl domain was mixed with either lipoamidase fraction or equivalent volume of buffer as control in a total volume of 100 µl, then 15 µl was removed and added to the E3/DTNB assay mix to initiate the reaction. The formation of TNB was followed for at least 45 s at 405 nm in triplicate to determine t=0 activity. This procedure was repeated for all the treatments of a given sample. The remaining lipoamidase fraction / lipoyl domain mixture was incubated at 37 °C for various times. At the end of the incubation time the aliquots of the samples were again assayed in triplicate (by initiating the E3/DTNB reaction with 15 µl incubation mixture). The rate data obtained was analyzed by using a two sample for means t-test for a significant difference between the t=0 and t=X rates. A significant difference in reaction rates between t=0 and t=X indicates lipoamidase removal of lipoic acid from the GST-LipDom. A treatment mix containing only lipoyl domain and buffer was included as positive control.

J. Purification

E. faecalis homogenates contain PDH, the lipoic acid activating system and lipoamidase. The first objective of the purification was to separate these three systems. Reed *et al.* (8) reported a procedure which separated the three components (Fig. II-8) using protamine sulfate to precipitate the PDH, followed by ammonium sulfate fractionation which separated the activating system from lipoamidase. I used this procedure unaltered. PDH typically precipitated with the addition of 0.16 - 0.20 volumes of 2% protamine sulfate. Addition of 0.35 g of solid ammonium sulfate per ml of solution typically completely precipitated lipoamidase, leaving the activating system in solution.

The partial purification of lipoamidase through DEAE column chromatography was done according to the protocol of Suzuki and Reed (31) and is outlined in Fig II-9.

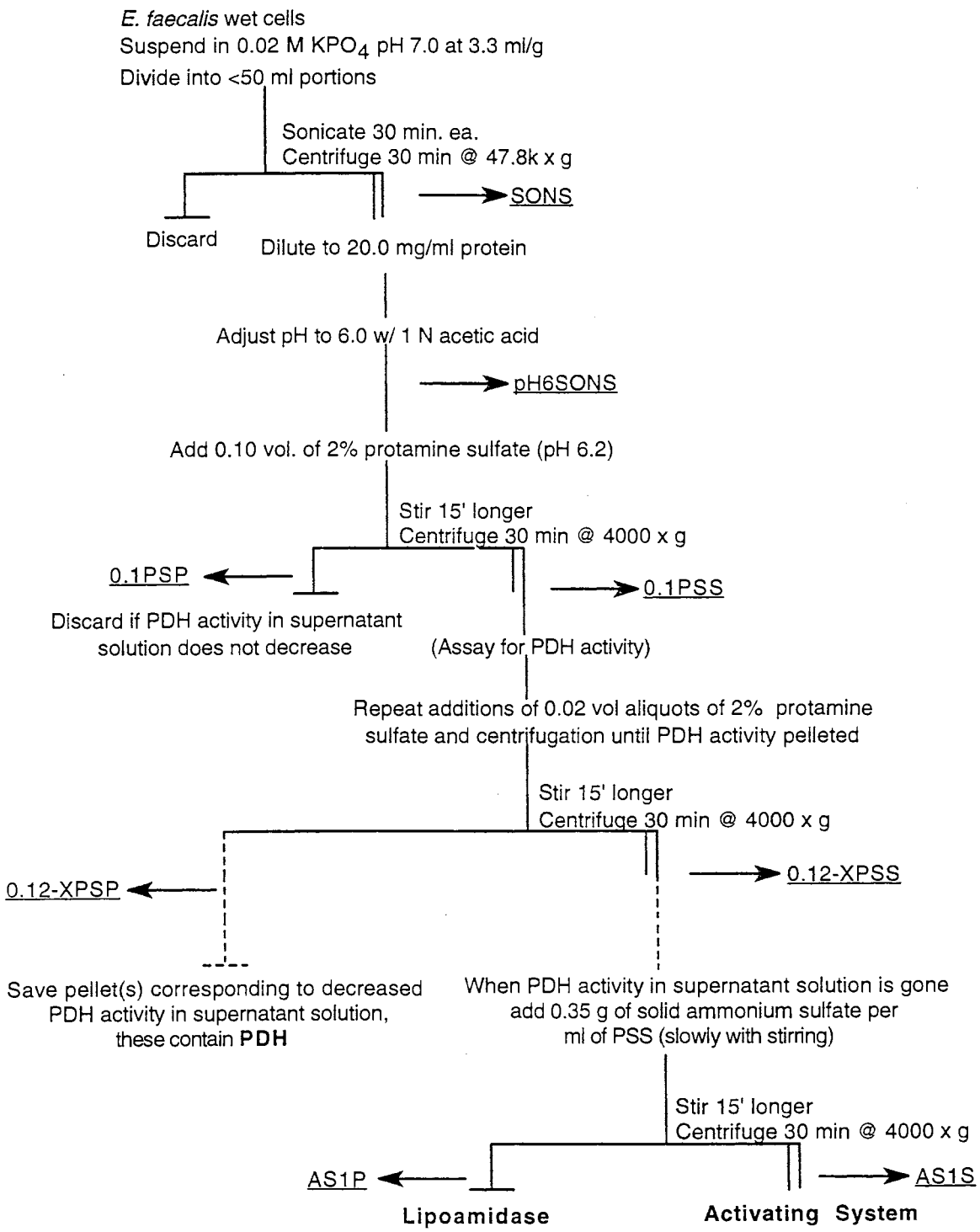

Figure II-8. Flow Chart for Separation of PDH, Lipoamidase and the Lipoic Acid Activating System. ‖ = supernatant solution, _|_ = pellet, dashed lines indicate operations which were repeated a variable number of times from batch to batch. PSP = protamine sulfate pellets, PSS = protamine sulfate supernatant solution, 0.12-XPSP and PSS indicate successive fractions as 0.02 volume portions of protamine sulfate are added, AS1P = ammonium sulfate pellet, AS1S = ammonium sulfate supernatant solution. →Fraction = fraction designation and that a sample is saved for assay.

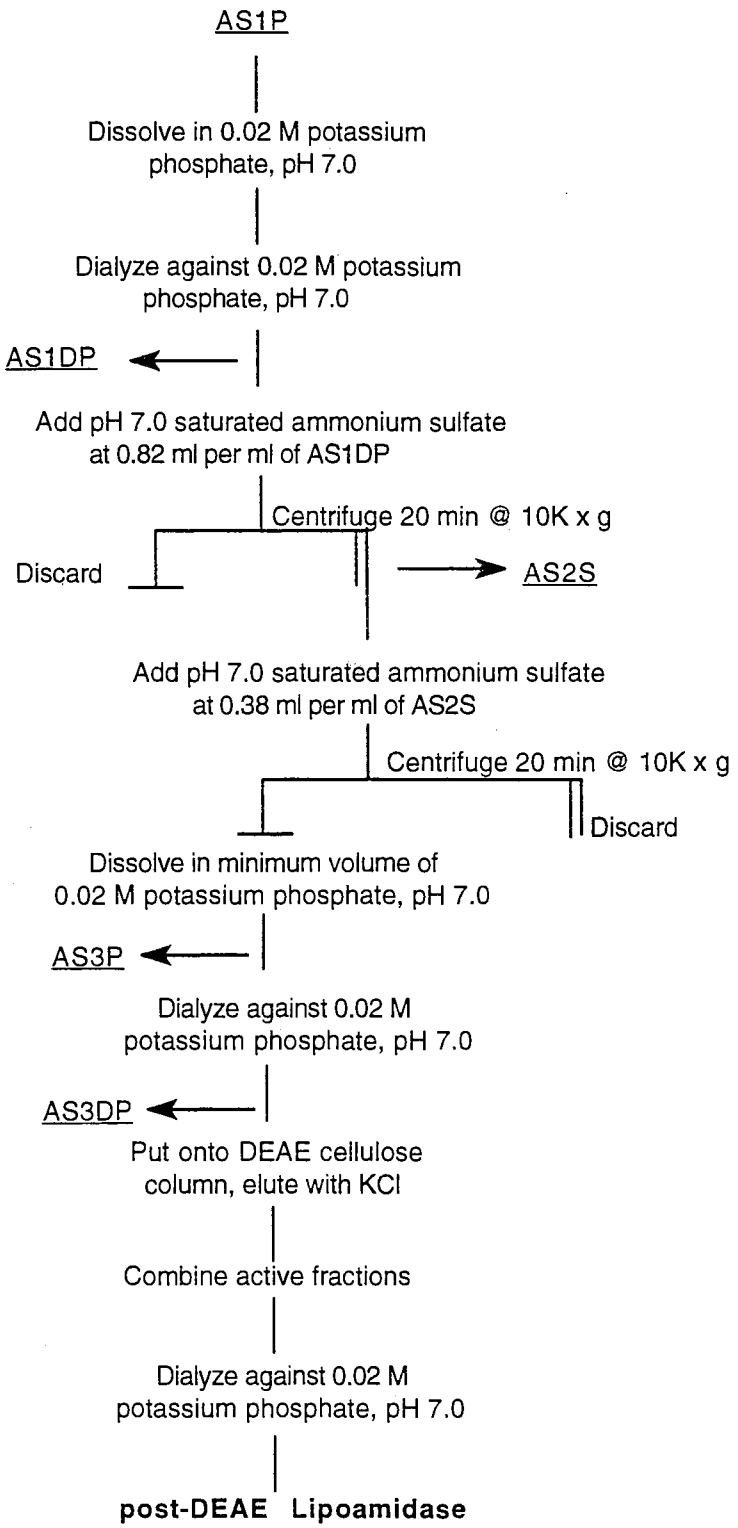

Figure II-9. Flow-Chart for Lipoamidase Purification Through DEAE. →Fraction indicate fraction designation and that a sample is saved for assay. Fractions designations: ending in 'P' indicate pellets, "DP" indicate dialyzed pellets and "S" indicate supernatant solutions.

K. Chromatography

1. DEAE column chromatography

A 2.5 cm × 12 cm column of Whatman DE-52 anion exchange resin was run using a Bio-Rad Econo pump, an Isco UA-5 detector and an Isco Foxy fraction collector. All operations were performed at 4 °C following the procedure of Suzuki and Reed (8) with the addition of a 0.5 M KCl column stripping step. The column was equilibrated in 20 mM potassium phosphate buffer, pH 7.0. The sample was applied and washed onto the column with 80 ml of starting buffer at a flow rate of 1.0 ml/min and monitoring at 280 nm while collecting eluent in an Ehrlenmyer flask. The column was then eluted with a stepwise gradient of KCl in starting buffer, monitoring protein elution at 280 nm and collecting 5 ml fractions in 13 × 100 mm test tubes. The steps were 80 ml each of 0.1 M KCl, 0.15 M KCl and 0.5 M KCl. Some column runs were washed with 80 ml of starting buffer between KCl steps. Fractions containing protein were assayed for lipoamidase activity [by the PDH inactivation assay or the LA-*p*ABA hydrolysis assay or the LipDom inactivation assay].

2. Hydroxylapatite column chromatography

Bio-Rad HTP and HTP-II 5 ml Econo columns were connected to a Bio-Rad Econo chromatography system. All operations were performed at 4 °C. The columns were equilibrated in 10 mM sodium phosphate buffer pH 7.2. Samples were applied to the column in 10 mM sodium phosphate, pH 6.8 and eluted in a gradient of 10-380 mM sodium phosphate, pH 6.8. Protein elution was monitored at 280 nm and fractions collected. The volume used for elution and fraction collection varied among column runs in order to optimize separation. Protein containing fractions were assayed for lipoamidase activity [as described for DEAE column chromatography].

3. Dye affinity chromatography

Sigma RDL-9 Dye Resin test kit columns (nine 2.5 ml bed volume columns each with a different dye attached to crosslinked beaded 4% agarose) were used. The dyes were Cibacron Blue 3GA (CB5), Reactive Green 5 (RG5-5), Reactive Brown 10 (RB10-5), Reactive Blue 72 (RB72-5), Reactive Blue 4 (RB4-5), Reactive Yellow 86 (RY86-5), Reactive Yellow 3 (RY3-5), Reactive Red 120 (RR120-5) and Reactive Green 19 (RG19-5). The Bio-Rad Econo System was used to pump, generate gradient, monitor and record elution A_{280} and collect fractions. All operations were performed at 4 °C. The columns were screened for lipoamidase retention as described below. The starting buffer was 20 mM potassium phosphate pH 7.0 and elution was achieved with KCl in 20 mM potassium phosphate, pH 7.0. The columns were equilibrated with 25 ml of starting buffer, and sample was loaded and washed on with another 25 ml of starting buffer at 0.8 ml/min while collecting the eluant in flasks. During screening the proteins were eluted in 1.5 M KCl in starting buffer while monitoring eluate at 280 nm and collecting 1.6 ml fractions. The flow-through and selected fractions were then assayed by the LA-*p*ABA assay in 96 well plates using 30 µmoles of LA-*p*ABA and 0.15 ml sample in 0.2 ml total volume and scoring by a visual presence or absence of pink color.

Later runs using columns which exhibited lipoamidase activity retention were eluted with linear gradients of 0-1.5 M KCl in starting buffer at 0.8 ml/min while monitoring at 280 nm and collecting 1.6 ml fractions. Protein containing fractions were assayed for lipoamidase activity with the LA- *p*ABA cleavage assay.

4. Mono Q anion exchange chromatography

A Pharmacia FPLC HR 5/5 Mono Q column was used with an Isco model 2360 gradient programmer, an LDC Analytical HPLC pump equipped with a pulse damper, an

Isco UA-5 detector and a Gilson model 203 fraction collector. All operations were performed at 4 °C . The column was equilibrated in BisTris, pH 7.0 ionic strength 0.01 ($I = 0.01$) starting buffer. Samples were loaded in starting buffer at 0.5 ml/min. Fractions were eluted in a linear gradient of 0.0-0.5 M KCl in starting buffer. Protein elution was monitored at 280 nm and 0.5 ml fractions were collected. Protein containing fractions were assayed for lipoamidase activity with the LA-*p*ABA cleavage assay and the LipDom inactivation assay.

5. Hydrophobic interaction chromatography

Two HIC columns were used: a Butyl Sepharose 4 Fast Flow, and an Octyl Sepharose 4 Fast Flow. Both columns were from a HiTrap™ HIC Test Kit from Pharmacia Biotech. The columns were equilibrated in 20 mM potassium phosphate buffer, pH 7.0 with 1 M KCl (starting buffer). The flow rate was 1.0 ml/min using a Bio-Rad Econo System for gradient formation, pumping, UV elution monitoring at 280 nm and fraction collection. The gradient involved a decrease in KCl from 1.0 M to 0 M. Samples were loaded in 1.0 M KCl, 1 ml fractions collected and protein containing peaks assayed for lipoamidase activity with the LA-*p*ABA hydrolysis assay and the LipDom inactivation assay.

L. Electrophoretic Methods

1. General

Standards used included Bio-Rad High Molecular weight standards (45-200 kDa), Bio-Rad Low Molecular weight standards (14-97 kDa), Sigma Mark VII standards (14-66 kDa) and Bio-Rad Kaleidoscope Pre-Stained standards (6.9-202 kDa).

Gels were stained with Coomassie Blue (53) or with Bio-Rad Silver Stain Plus as per supplier's instructions. When Coomassie Blue staining was unsatisfactory, gels were completely destained in methanol:acetic acid:H_2O (4:1:5), washed extensively in H_2O, and silver stained, omitting the silver fixative and subsequent wash step. Silver-stained gels exhibiting very high background were treated with a silver destaining solution as described by Bio-Rad until the background was satisfactory. If destaining was complete or bands disappeared, the gels were restained.

Analysis of gel band patterns was done using Bio-Rad Molecular Analyst software from gel images generated on a Bio-Rad densitometer

2. Phast System

Samples were often analyzed by SDS-PAGE on a Pharmacia Phast System™. Samples were prepared as recommended by Pharmacia. Gel formats used included 12.5% homogeneous, and 10-15% Gradient. Separation program conditions were essentially those recommended by the manufacturer. Coomassie Blue staining was done in the Phast System™ staining unit as per Pharmacia's instructions. Silver staining was done manually in a Petri dish with Silver Stain Plus reagents from Bio-Rad as per manufacturer's instructions using 1/5 the amount of reagents suggested for a mini gel.

3. Mini Gels

SDS and native PAGE gels were run with a Bio-Rad Mini Protean II apparatus. Buffers and solutions were as per Bollag and Edelstein (53).

For preparative native PAGE gels, multiple lanes were loaded with the fraction of interest. After the separation was performed the gel sandwich was carefully disassembled leaving the gel on one of the glass plates. The gel was then cut parallel to the direction of migration into sections for analysis. The sections were left together on the plate and

realigned as if the gel had not been cut. The section to be assayed for enzyme activity was then sliced into eleven or twelve pieces perpendicular to the direction of migration with a multiple razor blade gel slicer. Care was taken to make slice marks in the edge of the of gel section to be stained in order to correlate banding pattern to activity in slices (Fig. II-8).

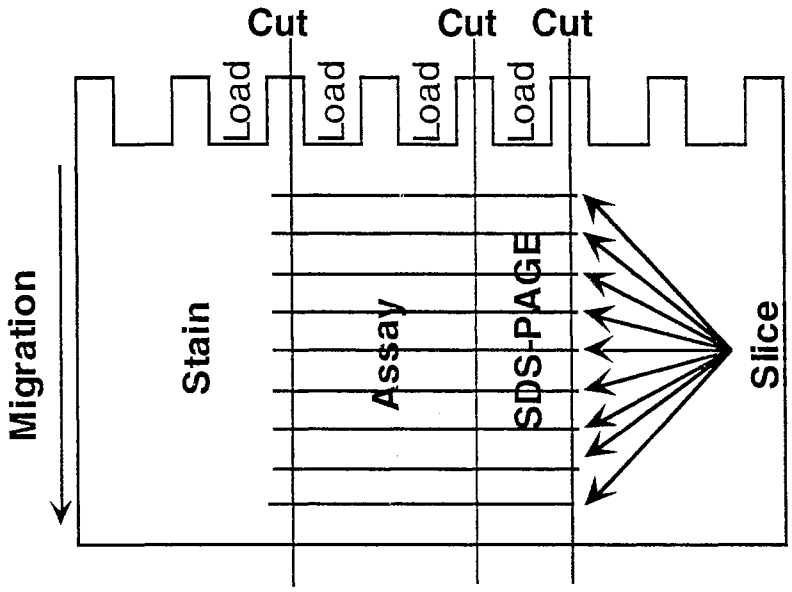

Figure II-10. Diagram of Preparative Native PAGE Gel. The "Stain" section was silver stained to give a native PAGE banding profile. The "Assay" section was utilized to obtain lipoamidase activity data from the electrophoretically separated proteins. The "SDS-PAGE" section was used obtain an SDS-PAGE profile of native PAGE separated proteins. Note slice marks left in "Stain" section to allow correlation of activity to banding pattern.

The gel slices to be assayed were treated in one of two ways. Each gel slice was put into 0.02 M potassium phosphate, pH 7.0 in a microfuge tube, ground with a pestle, soaked overnight at 4 °C, then centrifuged at full speed in a Beckman table top microfuge and the supernatant solution removed and added to assay mixture. The other method of treatment used electroelution with either a Bio-Rad electroeluter or a homemade device.

M. *Preparation of Protein for Microsequencing Using SDS-PAGE and Electro-transfer to PVDF*

1. Solutions

SDS-PAGE stock solutions

4x separating gel buffer - 1.5 M Tris, pH 8.8 with 0.4% SDS
4x stack gel buffer - 0.5 M Tris, pH 6.8 with 0.4% SDS
30% acrylamide - (30.8%T, 2.6%C)
10 mM sodium thioglycolate
Tank buffer - 25 mM Tris, 192 mM glycine and 0.1% SDS
10% ammonium persulfate
10% SDS
4X sample buffer (stored frozen) 20% glycerol, 28.8 mM 2-mercaptoethanol, 6% SDS, 0.005% bromophenol blue and 125 mM Tris, pH 6.
Pre-run Buffer - 0.38 M Tris, 0.1% SDS and 0.1 mM Na thioglycolate, pH 8.8
Stack set buffer - 0.5 M Tris, 0.1 mM Na thioglycolate, 0.4% SDS, pH 8.8
Stack set indicator - 0.008% bromophenol blue, 83% glycerol, 0.83% stack set buffer

Semi-dry electroblotting stock solutions

Transfer buffer [Bjerrum and Schafer-Nielsen (78)] 48 mM Tris, 39 mM glycine, 20% methanol and 1.3 mM SDS (pH should be ~8.2, if not the solution is remade).

Coomassie Blue Staining Reagents

G-250 Stain - 0.1% Coomassie Blue G-250, 40% methanol and 1.0% acetic acid (filtered through a 0.45 µm filter).
Destain - 50% methanol in H_2O

2. Procedure

Overview

LeGendre *et al.*, in "A Practical Guide to Protein and Peptide Purification for Microsequencing (pp74-100)" (55), identify N-terminal blockage that occurs during electrophoresis as a major problem in lowering N-terminal sequencing success. They

suggest pre-running the gel just prior to loading the sample. Promega, in "Protein Guide, Tips and Techniques" suggest polymerizing gels at least 4 h prior to sample loading and adding thioglycolic acid to the upper gel buffer. The protocol used takes all of these precautions as well as adding thioglycolate to the pre-run buffer to remove any unreacted gel components which might react with the N-terminal of proteins loaded and run on the gel.

Gel Preparation

This protocol used a single buffer (separating gel buffer) for separating and stacking gel since the prerun with Na thioglycolate would abolish a normal buffer discontinuity. The separating gel solution was prepared without APS and TEMED and degassed for 5 min under vacuum. TEMED and APS were added and the solution poured to within 2.0 cm of the top of the short plate, carefully overlaid with H_2O and allowed to polymerize for one h. The water was then poured off and the top of the gel rinsed with water. Degassed 4% stacking gel solution was then added, the appropriate comb set in place, and the gel allowed to polymerize overnight at 4 °C.

Prerun and pH Reset of Stacking Gel

The gel was pre-run with pre-run buffer at 50 V for one hour at 4 °C to prevent artifactual N-terminal blockage of electrophoresed proteins. The samples to be run were mixed 2:1 with sample buffer and boiled for 2 min. After the pre-run the buffer was poured out of the inner buffer chamber and the wells rinsed thoroughly with H_2O. The unit was reassembled and the inner buffer chamber was filled with Stack Set buffer. Stack Set indicator (10 µl) was put into each of the outside and a middle well and the unit was run at 50 V until bromophenol blue reached the stacking/separating gel interface.

Sample Loading and Gel Run

After resetting the stacking gel both buffer chambers were emptied and gel wells were thoroughly rinsed with H_2O. The buffer chambers were refilled with tank buffer and 1.5 ml of 10 mM Na-thioglycolate was added to the upper tank and stirred. An outside lane was loaded with Kaleidoscope™ prestained standards. The samples were loaded and electrophoresed at 25 mA.

Transfer, Staining and Band Recovery

While the gel was running a 5.5 X 8.0 cm piece of PVDF membrane was wet in 100% methanol for a few seconds and then equilibrated for at least 15 min in transfer buffer. Two pieces of 5.5 X 8.0 cm Bio-Rad transfer filter paper were also soaked in transfer buffer. Electrophoresis was stopped when the dye front neared the bottom of the gel. The gel was removed from the electrophoresis unit and soaked for five min in 100 ml of transfer buffer. The transfer apparatus (Bio-Rad SD semi-dry) was assembled with gel in place according to manufacturer's instructions. After electroblotting at 15 V for 15 - 30 min the current was stopped and the cathode plate was carefully lifted without disturbing the gel/membrane sandwich. Then the membrane was carefully separated from the gel at the end containing the pre-stained standards and an evaluation of the transfer was made. If the prestained standards were still in the gel the sandwich was resealed, the cathode replaced and the transfer continued. When the transfer was complete, the unit was disassembled and the membrane was rinsed with H_2O. The membrane was then soaked in 100% methanol for a few s and placed into Coomassie Blue G-250 stain and incubated with orbital shaking. Protein bands typically appeared within one min. Longer staining time resulted in very high background. Moderate background was removed by soaking in destain solution until background was acceptable. The membrane was then rinsed thoroughly with H_2O and the band of interest was cut out with a clean razor blade. The band alone was further rinsed with H_2O to remove all traces of transfer, staining and

destaining buffers. The gels were also stained to visualize proteins left in the gel and to analyze transfer efficiency.

CHAPTER III

RESULTS

A. Overview

The purpose of this work was to obtain pure lipoamidase. Since the Reed and Suzuki (8) procedure achieved a 61-fold purification with a 46% yield, I started by using their procedure. For protein that has been purified through the DEAE column fractionation, I sought additional purification by measuring specific activity and by determining the number of protein species using PAGE.

Fractions from hydroxylapatite chromatography were obtained with lipoamidase activity and constitute ~200-800 fold purification with three proteins present. The three proteins were sequenced and two of them were fragments of elongation factor Ts (EF-Ts). The third protein has similarities to three different dehydrogenase sequences and also a glucosidase. This protein also has an NAD^+-binding domain. Ion exchange chromatography on a MonoQ anion exchange column and a CM cation exchange column did not further separate the three proteins. The three proteins were not resolved nor significantly purified by hydrophobic interaction chromatography, dye affinity chromatography or preparative native PAGE. Three different assays were employed over the course of purification with none of the assays found to be well suited for anlayzing the purification of lipoamidase. One of the standard assays for mammalian lipoamidase

(cleavage of Lipoyl-*p*ABA to lipoic acid and *p*ABA) is not specific for lipoamidase activity in *E. faecalis.*

B. Initial Results

I began purifying lipoamidase using the procedure of Reed and Suzuki (8) (Fig. II-7). *E. faecalis* cells were broken by sonic oscillation and PDH was then precipitated with protamine sulfate. Lipoamidase and the activating system were separated by precipitating lipoamidase with ammonium sulfate. The lipoamidase was then purified with two more ammonium sulfate fractionation steps. The last step in the Reed and Suzuki purification of lipoamidase was a DEAE cellulose anion exchange column with a three step gradient consisting of sample application in 0.02 M KPO_4, a 0.10 M KCl in 0.02M KPO_4 elution, and a final elution with 0.15 M KCl in 0.02 M KPO_4. The lipoamidase activity, as followed by the PDH inactivation assay, eluted with the 0.15 M KCl step. I added a 0.50 M KCl in 0.02 M KPO_4 wash following the 0.15 M KCl elution to clean the column. My first 3 purifications, done before early 1994, agreed with those of the Reed group in that lipoamidase activity eluted in the 0.15 M KCl fractions. SDS-PAGE with Coomassie Blue staining of pooled active fractions showed a major band at ~36 kDa and at least 2 minor bands below 20 kDa (Fig. III-1).

After April 1994, DEAE chromatography yielded A_{280} patterns which looked nearly identical (Fig. III-2) to previous patterns, but lipoamidase activity was found in both the 0.15 M KCl eluant and in the 0.10 M KCl eluant. Difficulties with salt interference in the PDH inactivation assay led me to look for an assay which would be less sensitive to salt concentration. An assay using lipoic acid attached to *p*-amino benzoic acid (LA-*p*ABA) as a substrate (see Ch. II) proved less sensitive to salt concentration than the PDH inactivation assay. With the new assay in hand, I proceeded with purification up through DEAE chromatography (elution profile not shown). When DEAE fractions were assayed the major LA-*p*ABA cleaving activity was found in the 0.10 M KCl peak with less activity in

the 0.15 M KCl and some in the 0.5 M KCl wash. The active fractions in the 0.10 M KCl peak were combined, concentrated and then re-assayed along with previous fractions using the LA-*p*ABA assay to calculate purification data (Table III-1).

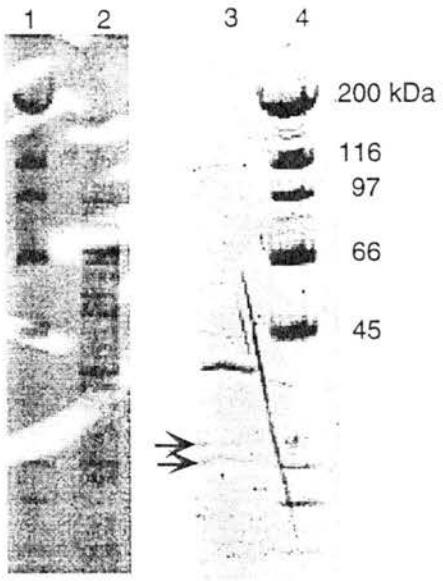

Figure III-1. SDS-PAGE of Combined DEAE Fractions That Contain Lipoamidase. Fractions 31-33 from DEAE anion exchange chromatography were combined and concentrated by ultrafiltration and a portion subjected to SDS-PAGE as described in Materials and Methods. Lanes 1 and 4 are Bio-Rad High Molecular Weight Markers. Lane 2 is AS3DP fraction [see purification scheme, Fig. II-9]. Lane 3 is pooled active DEAE fractions 31-33 which were dialyzed against 20 mM potassium phosphate buffer, pH 7.0 and concentrated. Minor protein bands clearly visible on the original gel are not visible on this reproduction but are indicated by arrows. The major band is ~36 kDa and comprises >90% of total Coomassie Blue stained protein. Two minor bands are < 20 kDa and comprise ~5% each of total Coomassie stained protein.

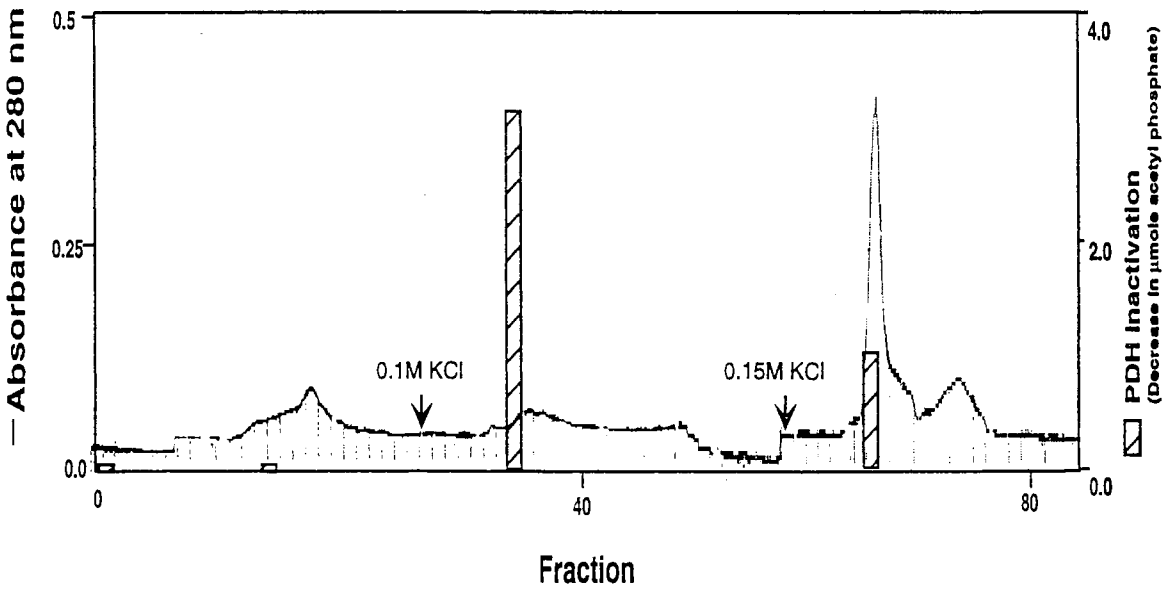

Figure III-2. DEAE Column Chromatography. Post April, 1994 DEAE column run showing PDH inactivation activity in the 0.1 M KCl and 0.15 M KCl fractions.

Table III-1. Lipoamidase Purification Table Based on LA-*p*ABA cleavage assay. Crude extract is post sonication supernatant solution, pH 6.0 SONS is the pH adjusted extract, 0.2 Prot Sulf is the extract after PDH had been removed by protamine sulfate precipitation, AS1DP is the dissolved dialyzed pellet after the first ammonium sulfate precipitation, AS3DP is the dissolved dialyzed pellet after the third ammonium sulfate precipitation, DEAE is combined active fractions from the 0.10 M KCl eluate.

Fraction	Specific Activity (nmole/min·mg)	% Yield	Purification
Crude extract	0.73	100.0	1.0
pH 6.0 SONS	0.80	81.9	1.0
0.2 Prot Sulf	3.01	59.1	4.1
AS1DP	7.16	50.9	9.8
AS3DP	5.41	15.7	7.4
DEAE	22.00	7.7	30.2

Reed and Suzuki (31) reported 61-fold purification with 46% yield through the DEAE step using the PDH inactivation assay. Using the LA-*p*ABA assay I obtained only 30-fold purification and less than 10% yield from the most active of the two peaks from the DEAE step. Based on the poor purification and the presence of activity in multiple fractions, the 0.10 M KCl fractions were assayed with the PDH inactivation assay to verify the presence of lipoamidase activity. There was no inactivation of PDH in the 0.10 M KCl pooled fraction but the 0.15 M KCl fractions inactivated PDH. Calculation of purification data using the PDH inactivation assay (Table III-2) showed a 58-fold purification and 10.5% yield from the 0.15 M KCl peak. This was closer to the Reed and Suzuki results.

Table III-2. Lipoamidase Purification Table Based on the PDH Inactivation Assay. Not all fractions assayed using LA-*p*ABA were assayed here. pH 6.0 SONS is the pH adjusted extract, AS1DP is the dissolved dialyzed pellet after the first ammonium sulfate precipitation, AS3DP is the dissolved dialyzed pellet after the third ammonium sulfate precipitation, DEAE is the combined active fractions from the 0.15 M KCl eluate.

Fraction	Specific Activity (nmole/min·mg)	% Yield	Purification
pH 6.0 SONS	9.63	100.0	1.0
AS1DP	66.46	47.9	6.9
AS3DP	102.4	30.0	10.6
DEAE	553.8	10.5	58.0

Further analysis verified the presence of lipoamidase activity in the 0.15 M KCl fraction; there was 89% inactivation of a holoPDH preparation and then a 72% recovery by incubation with an activating system preparation (Fig. III-3).

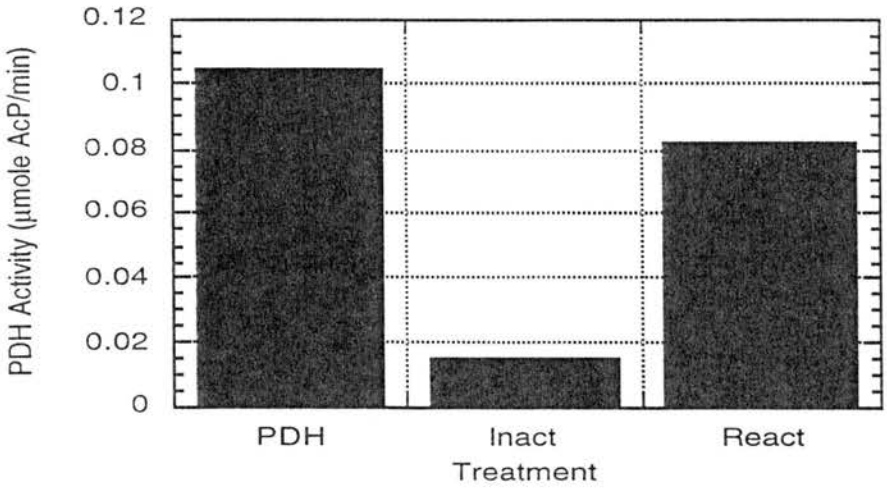

Figure III-3. Inactivation of PDH by Lipoamidase with Reactivation. The "PDH" set was the positive PDH control in which only buffer was added during the stage 1 and stage 2 incubations (see Materials and Methods). The "Inact" set was the PDH activity after incubation with 0.15 M KCl post-DEAE lipoamidase fraction. The "React" set was the PDH activity of an aliquot of the "Inact" set after incubation with activating system.

SDS-PAGE analysis (Fig. III-4) of the 0.15 M KCl fractions showed 6 bands with the most prominent at ~ 35 kDa (~56 %) the next at ~32 kDa (~22%) and minor bands (~9% and 12%) smaller than 30 kDa. When the gel was first developed there were other very faint bands which either faded with storage or are too faint to be seen in the picture.

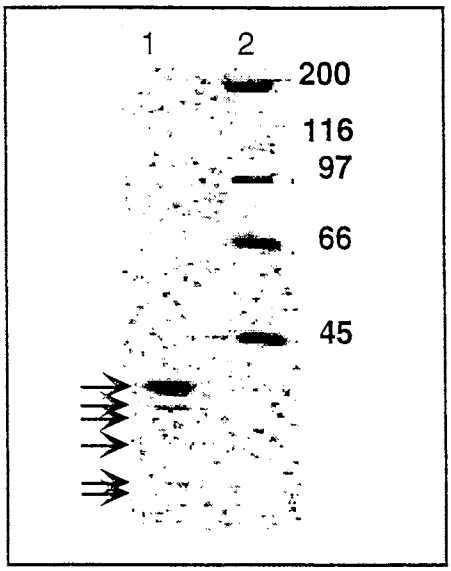

Figure III-4. Silver Stained SDS-PAGE of 0.15 M KCl DEAE Eluate Pooled Active Fractions. Lane 1 is 0.5µg of pooled active fractions from the 0.15 M KCl eluate which had been dialyzed against 20 mM potassium phosphate buffer pH 7.0. Not all of the bands evident on the gel are visible in this picture but are represented by arrows. Lane 2 is Bio-Rad High Molecular Weight Markers.

The next time a DEAE column was run I used the LipDom inactivation assay (E3/DTNB) acquired from Dr. Thomas Roche. I used the LA-*p*ABA assay to do initial screens on column fractions and then used the LipDom inactivation assay to establish lipoamidase activity. LA-*pABA* cleavage activity was found in the 0.02 M KPO_4 fraction and in the 0.15 M KCl fractions. However the LipDom inactivation assay showed activity only in the 0.15 M KCl peak (Fig. III-5). Different results were obtained from subsequent DEAE columns in that LA-*p*ABA cleavage activity was in the 0.02 M KPO_4, 0.10 M KCl, 0.15 M KCl and the 0.50 M KCl eluant. These two runs differ from all previous ones in that LA-*p*ABA cleavage and LipDom inactivation activity were found in both the 0.10 M and 0.15 M KCl eluants (Fig. III-6).

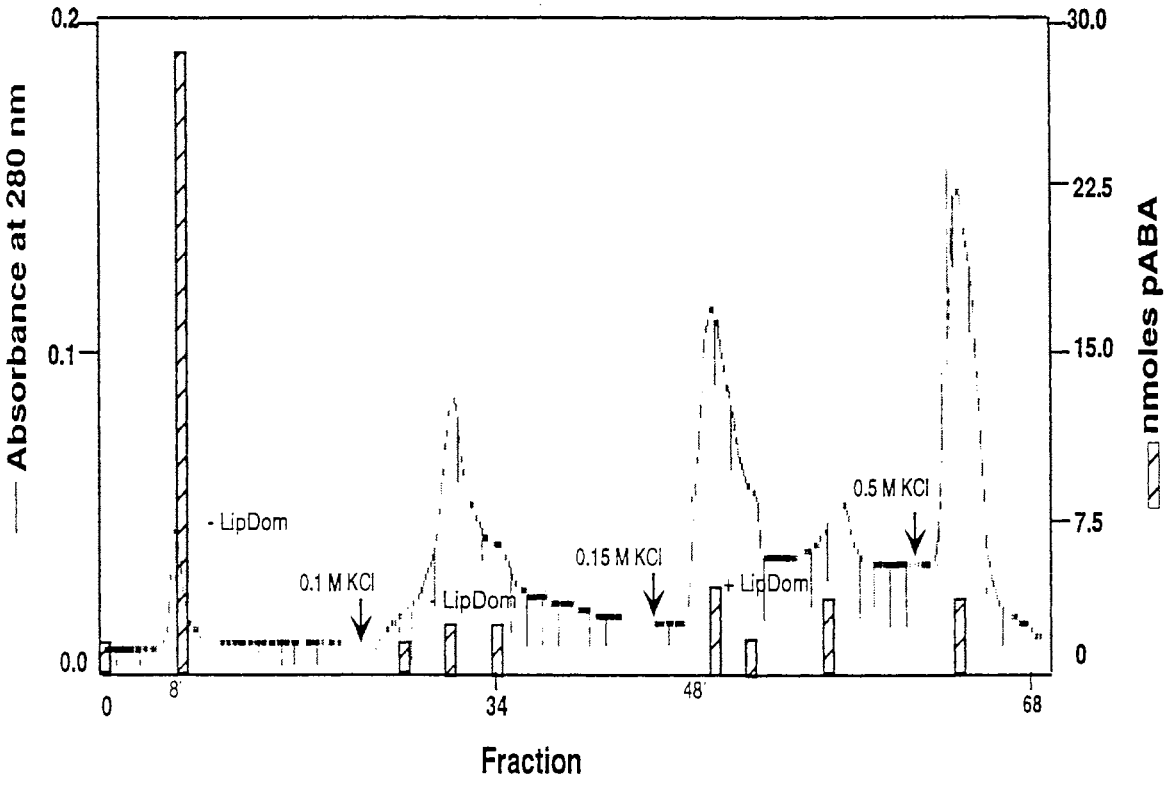

Figure III-5. DEAE Chromatogram with LA-*p*ABA Cleavage and LipDom Inactivation Activity. Solid bars represent LA-*p*ABA cleavage activity as nmoles of *p*ABA generated. LipDom inactivation activity is indicated by +/- LipDom, fraction 8 reduced LipDom activity a statistically non-significant 4.6 nmole/min·mg (t-stat =1.57), fraction 48 reduced LipDom activity a significant 14.0 nmole/min·mg (t-stat = 6.8, the t-Critical for P=.05 is 2.9).

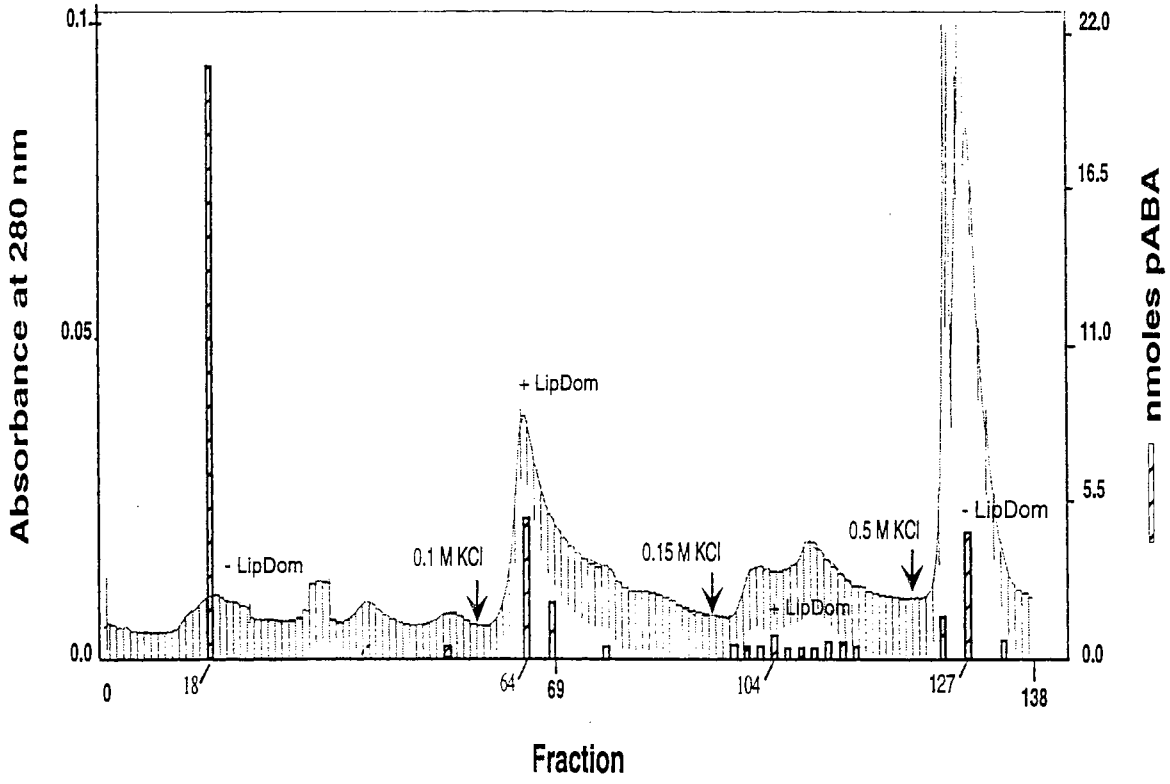

Figure III-6. DEAE Chromatogram with LA-*p*ABA and LipDom Cleaving Activity in 0.1 M KCl eluant. Bars represent LA-*p*ABA cleavage activity as nmoles of *p*ABA generated. LipDom inactivation is represented by +/-LipDom and plain text fraction numbers indicate fractions assayed for LipDom inactivation. The 0.02 M KCl eluate did not inactivate LipDom (t-stat = 0.04), fraction 64 inactivated LipDom a statistically significant 28.2 nmole/min·mg (t-stat = 8.49), and fraction 104 inactivated LipDom a statistically significant 14.5 nmole/min·mg (t-stat = 10.71, the t-Critical at P=.05 is 2.9).

I found that screening DEAE column fractions with the PDH inactivation assay often gave false positives because the salt concentration of the elution buffer was high enough to inhibit PDH activity. Therefore I began using LA-*p*ABA to assay column fractions because it is a relatively fast and easy assay and is not affected by high salt concentration. However it is not very specific as some fractions with high LA-*p*ABA cleaving activity are unable to inactivate PDH. Therefore the assay was used for initial screening of fractions and the positive ones were then assayed with another method to verify lipoamidase activity. The LipDom inactivation assay is not sensitive to the salt concentration and was often used to verify lipoamidase activity. In later runs LipDom inactivation (lipoamidase) was found not only in the 0.15 M KCl eluant but also in the 0.10 M KCl fraction.

C. Native PAGE

Attempts were made using non-denaturing polyacrylamide electrophoresis to purify lipoamidase. Clean banding patterns were reproducibly obtained without any special techniques other than omitting SDS, 2-mercaptoethanol and boiling the sample. Both pre-DEAE and post-DEAE fractions were used and activity was assayed using the LA-*p*ABA hydrolysis and PDH inactivation assays. Some gels had multiple peaks of activity with both assays in gel slices but the two assays often were not in agreement.

One attempt at native PAGE (NP5) involved loading pooled active DEAE fractions. No protein bands were found in the first five slices by silver staining. Slices #6, #7, #8, #9, and #11/12 (took the bottom of #11 and the top of #12 based on the stained lane) were electroeluted and assayed for PDH inactivation and reactivation activity. The results are shown in Fig. III-7.

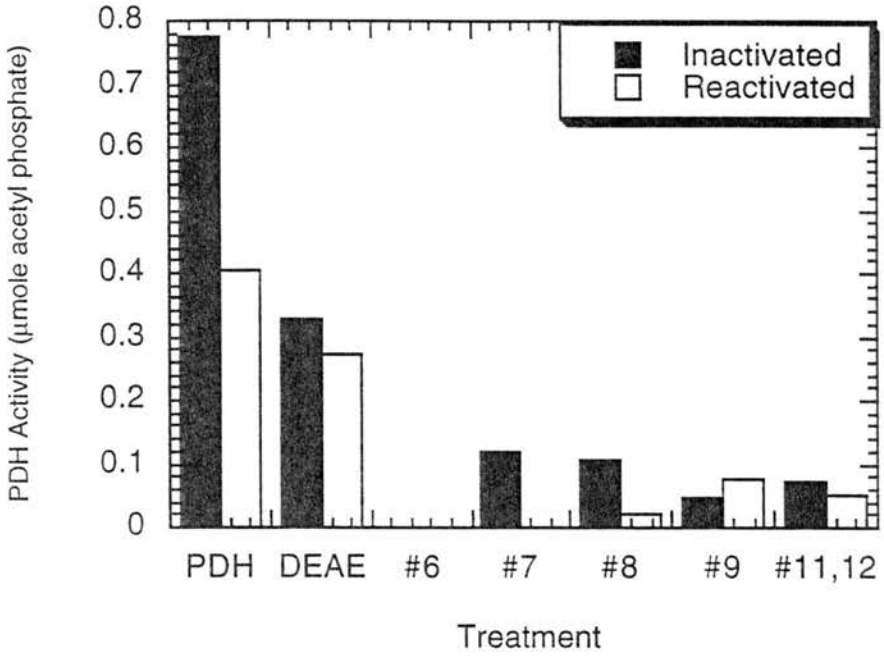

Figure III-7. Lipoamidase Activity Assay of NP5 Native PAGE Gel Slice Eluants by PDH Inactivation/Reactivation. Micromoles acetylphosphate is a measure of PDH activity. The activating system control activated > 3 µmole of PDH activity. The "inactivated set was simply apo-PDH and "reactivated" set was apo-PDH plus activating system. PDH indicates a control with no lipoamidase fraction added in stage one (activating system was added in stage two). DEAE indicates pooled active fractions from DEAE anion exchange chromatography which were loaded on the native gel. #6 - #11,12 indicate slices from the native gel. Inactivated sets are those which had holoPDH incubated with the indicated treatment in the first stage of the assay and buffer added in the second stage. Reactivated aliquots are those which had holoPDH incubated with the indicated treatment in the first stage of the assay and lipoic acid activating system added in the second stage.

Note that the DEAE treatment and all gel slices showed reduced PDH activity from the control level and incubation with activating system resulted in a loss of activity with the PDH control and further loss of PDH activity in all treatments except slice #6 and #9. Also note that activating system control was positive where treatment of apoPDH with lipoic acid and activating system resulted in more than 3.0 micromoles of acetylphosphate generated above a baseline of 0. Slice #9 showed a very slight recovery of PDH activity which does not seem significant but since activating system further reduced PDH activity in PDH

positive control, slice #7, #8, and #11/12 it may be significant. Slice #9 is also probably the same as slice #10 from a previous gel (data not shown). Slice #9 was analyzed by SDS-PAGE and contains only one band of ~36 kDa. This corresponds to the major band in the best DEAE preparation. The protein in the slice #9 eluant was TCA precipitated and the pellet washed in preparation for sequencing. The prepared protein was submitted to the Peptide and Protein Sequencing Facility of the Core Facility at Oklahoma State University. No sequence data were obtained.

Another attempt at obtaining activity from native gel slices (NP6) was made with a DEAE active fraction using only the LA-*p*ABA cleavage assay. Only slices 10-12 showed any protein and these were assayed. Slice #10 generated 3.33 nmoles of *p*ABA while slices #11 and #12 generated 0.54 and 0.21 nmoles respectively. Extracts from slice #10 and #11 were analyzed by SDS-PAGE and both gave a major band at ~35 kDa comprising >90 of the silver stained protein.

Several attempts were made to use a homemade continuous elution electrophoresis apparatus and a Bio-Rad Prep Cell under native conditions with no success.

Fractions from an HTP column with LipDom inactivation activity were loaded on a native gel, sliced and assayed for LipDom inactivation activity. Activity data was not obtained due to technical difficulties but SDS PAGE profiles of the slices were obtained.

D. *Hydroxylapatite Column Chromatography and Subsequent N-terminal Sequencing*

Hydroxylapatite chromatography was used in an attempt to purify lipoamidase after DEAE anion exchange chromatography. With the first two columns (HTP1 and HTP2), fractions were assayed by the PDH inactivation assay only. All other runs used the LA-*p*ABA assay to screen fractions and then the LipDom inactivation assay to establish lipoamidase activity.

In the first column run (HTP1) pooled active DEAE fraction was loaded on the column in 10 mM sodium phosphate. The A_{280} trace showed only a rise above baseline in

the flow through eluant and a slight rise off of baseline at ~300 mM sodium phosphate (data not shown). Fractions from those regions were assayed with the PDH inactivation assay. Fig. III-8 shows that the 400 mM elution buffer only control reduced PDH activity from 1.78 μmoles acylphosphate to 1.44 μmoles and the lipoamidase preparation loaded on the column (DEAE) reduced it to 1.08 μmoles. No decrease in PDH activity was seen with fractions from the 10 mM sodium phosphate flow through but fraction 24 and 25 (~250-300 mM sodium phosphate) reduced PDH activity from 1.78 μmoles acylphosphate to 0.86 and 0.85 μmoles respectively.

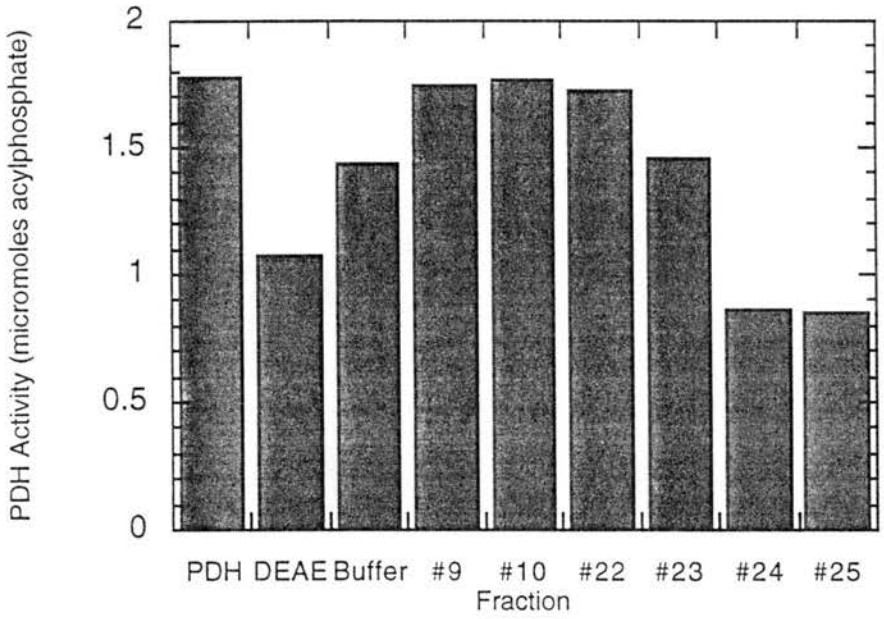

Figure III-8. PDH Activity Inactivation with HTP1 Fractions. "PDH" is the no lipoamidase control, "DEAE" is a control of the preparation which was loaded on the HTP column. "Buffer" was the 400 mM elution buffer only control. Fraction 9 and 10 are from the 10 mM $NaPO_4$ eluate and fraction 22, 23, 24 and 25 were from eluate at ~ 250-300 mM sodium phosphate.

The column was loaded with another aliquot of DEAE purified lipoamidase and run again (HTP2) with the sensitivity of the detector increased (Fig. III-9). This run produced

wide peaks roughly corresponding to the small peaks seen in the first run (HTP1) with large peaks in the 10 mM sodium phosphate and at ~ 170 mM sodium phosphate with a shoulder at ~200 mM. Selected fractions were analyzed by the PDH inactivation assay. There is a general trend of increasing PDH inactivation with increasing sodium phosphate concentration. The exception is with fractions 26-28 where there is a peak of PDH inactivation which does not follow the buffer gradient.

Fractions 23-25 of HTP1, which inactivated PDH, were pooled. Fractions 24-26 and fraction 27-29 of HTP2 were pooled based on elution A_{280} pattern and PDH inactivation. The protein concentration of the pooled active fractions was determined and specific activity calculated (Table III-3). Aliquots of each pooled fraction containing 7.2 μg of protein were concentrated to approximately 10 μl by ultrafiltration. These samples were then subjected to SDS-PAGE and transferred to PVDF as described under "Preparation for N-terminal Microsequencing" in Chapter II (Methods and Materials). The resulting Coomassie Blue stained PVDF membrane had what appeared to be three identical bands in each of the sample lanes with differences in relative concentration (Fig. III-10, Table III-3).

Calculation of molecular weight using Sigma Mark VII standards indicated band I to be ~38,000 Da, band II to be ~35,000 Da and band III to be ~32,000 Da. The bands from the HTP2 fraction 27-29 (lane 4) were cut out and sequenced. Band I yielded a sequence of nine amino acids (Fig. III-11). Band II gave a 30 amino acid sequence(Fig.III-11) and band III yielded 16 amino acids which were of questionable accuracy due to sequencing instrument error. Later band III from HTP2 fractions 24-26 was analyzed and fifty-eight amino acids of sequence (Fig. III-11) were determined. Analysis of these fifty-eight amino acids revealed that the initial band III sequencing attempt from HTP2 27-29 best guess matched the first sixteen amino acids. This confirmed that the bottom band in the three HTP preparations is the same and strengthens the case that all three bands in all three lanes are the same.

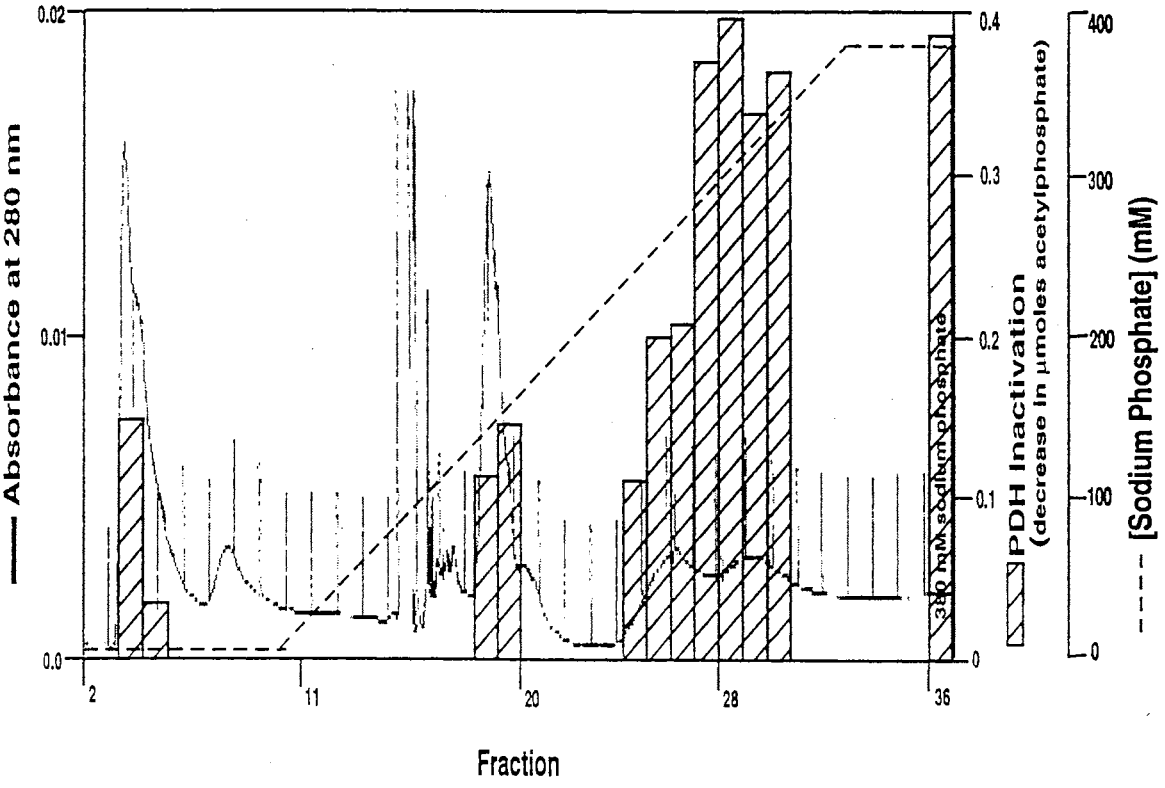

Figure III-9. HTP Chromatography (HTP2) with PDH Inactivation Activity. Hatched bars indicate PDH inactivation. The bar labelled 380 mM sodium phosphate is the elution buffer control. The dashed line indicates the sodium phosphate elution gradient.

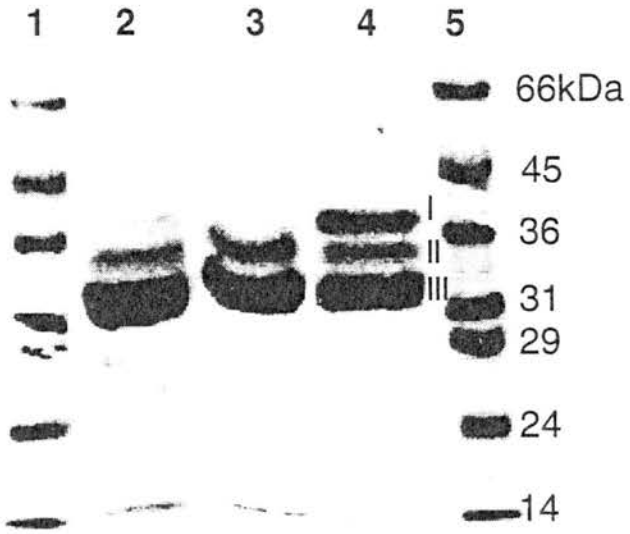

Figure III-10. SDS-PAGE of Hydroxylapatite Fractions. This is a Coomassie Blue stained PVDF blot. Each lane was loaded with 12 µg of protein. Bands labeled I, II, and III from lane 4 were cut from this blot and sequenced. Lane 1 and 5 are Sigma molecular weight markers. Lane 2 is HTP1 pooled fraction 23-25 which has lipoamidase activity, lane 3 is HTP2 pooled fraction 24-26 which has low lipoamidase activity and lane 4 is HTP2 pooled fraction 27-29 which has lipoamidase activity. Bands corresponding to band I in lane 4 were also present in lanes 2 and 3 but they are not evident in this picture.

Table III-3. Relative Amounts of Protein in Bands from Pooled HTP Fractions and Fraction Activity. Molecular weight is reported in Daltons and was calculated based on Sigma Mark VII standard migration. Stain % is based on band density in each lane. The µg values are from 7.2 µg of protien loaded and calculated from Stain %. Activity is PDH inactivation in µmole/min·mg for the pooled fraction.

	HTP1 23-25 Stain% / µg	HTP2 24-26 Stain% / µg	HTP2 27-29 Stain% / µg	Molecular Weight
Band I	**15% / 1.1**	**10% / 0.72**	**30% / 2.1**	**~38,000**
Band II	**25% / 1.8**	**30% / 2.16**	**20% / 1.4**	**~35,000**
Band III	**60% / 4.3**	**60% / 4.32**	**50% / 3.6**	**~32,000**
Activity	**9.5**	**6.6**	**25.8**	

Band I

ADVTAKMVK

Band II

MKIAIAGAGA MGSRFGLMLK QGRNDVLLII

Band III

KKNDRIAAEG LANVATVNGV AAIVEVNSET DFVSKNEMFQ

DLVKDIATKV AKNDPATM

Figure III-11. Amino Acid Sequence of HTP Pooled Fraction Proteins. Band I is the ~39 kDa band, Band II is the ~35 kDa band, Band III is the ~31 kDa band.

At a later date a ~36 kDa band from a fraction with LipDom inactivation activity from a MonoQ Anion Exchange column yielded the 28 amino acid sequence shown below.

~36 kDa band from MonoQ

ADVTAKMVKE LRDMTGVGMM DAKKCLVK

The first 9 amino acids are the same as band I from the HTP column fraction. A search of all 28 amino acids against the GenBank CDS database using a BLAST+BEAUTY server at the Human Genome Center, Baylor College of Medicine identified HTP band I as the first 28 amino acids of elongation factor Ts (EF-Ts). Compared to EF-Ts from *Streptococcus pnuemoneae* there is 62% identity with 88% similarity across the 28 residues. Searches against other data bases with different search tools also gave the best matches with EF-Ts sequences.

A BLAST+BEAUTY driven search of the GenBank CDS data base through the National Center for Biotechnology Information Blast server gave the best match between all 58 amino acids of band III and amino acid 51-108 of EF-Ts from *Spiroplasma citrii* with 50% identity and 67% similarity across the 58 residues. Based on the results of the

sequence database searches with the band I and band III sequences, the two were combined assuming band I is the N-terminus and band III is amino acid 51-108. The resulting chimera was aligned with prokaryotic EF-Ts sequences as shown in Fig. III-12.

```
ECOLI   1 maEITASLVKELRERTGAGMMDCKKALTEangdielaienmrksgaik-
HAEIN   1 maEITASLVKELRDRTGAGMMECKKALVEangdielaidnmrksgqak-
STRPN   1 x-EITAKLVKELREKSGAGVMDAKKALVE 28----------------
SPICI   1 m-EVTAQLVKELRDRTGAGMLDCKKALEDslagilkkqlhgyvqkgitk
Efae      a-DVTAKMVKELRDMTGVGMMDAKKCLVK 28xxxxxxxxxxxxxxxxxx

ECOLI     aaKKAGNVAADGVIKTKIDGNYGIILEVNCQTDFVAKDAGFQAFADKVL
HAEIN     aaKKAGRVAAEGVILARVENGFGVLVEMNCETDFVAKDAGFLGLANEVT
STRPN     --------------------------------------------------
SPICI     aaKKSDRVAAEGLVGLVTKGDKQIIFEVNSETDFVAKNKQFKDLMATVG
Efae      xxKKNDRIAAEGLANVATVNGVAAIVEVNSETDFVSKNEMFQDLVKDIA

ECOLI     DAAVAGKITDV 108
HAEIN     DFAAANKGTTI 108
STRPN     ----------
SPICI     ETLINNDPKTV 108
Efae      TKVAKNDPATM
```

Figure III-12. Alignment of Sequenced Bands with EF-Ts. ECOLI is partial sequence of EF-Ts from *E. coli* sp# P02997, HAEIN is partial sequence of EF-Ts from *Haemophilus influenzea* sp# P43894, STRPN is all of the known sequence of EF-Ts from *Streptococcus pnuemonea* sp# P80715, SPICI is EF-Ts from *Spiroplasma citrii* sp# P19216. Amino acids in bold are those which are either identical or similar in all 5 sequences.

Band I and band III contain two EF-Ts signature sequences which are found in the N-terminal region of EF-Ts proteins. Fig. III-13 shows the standard notation with relevant band I and band III sequences and Fig. III-14 shows the signature sequences aligned against the combined band I and band III. The EF-Ts signature sequences do not represent any known functional motiffs but are sequences conserved in all known EF-Ts proteins and are not found in other proteins.

PS01126 **L-R-x(2)-T-[GDQ]-x-[GS]-[LIVMF]-x(0,1)-[DENKAC]-x-K-[KRNEQ]-A-L**
Band I) **L-R-D-M-T--G------V- -G---------M-----M------D------------A-K--K----------C-L**

PS01127 **E-[LIVM]-N-[SCV]-[QE]-T-D-F-V-[SA]-K-R-N**
Band III) **E-----V-----N--S----------E--T-D-F-V --S----K-R-N**

Figure III-13. EF-Ts Signature Sequences with Sequence from Band I and Band III. *PS01126* is the EF-Ts signature sequence and Band I and III represent the band I and band III sequences from hydroxylapatite chromatography. Amino acids in [] represent variations at the given position, numbers in () represent the number of amino acids allowed, x indicates any amino acid is allowed. EF-Ts signature sequences and notation are from PROSITE: PDOC00867 at http://expasy.hcuge.ch/cgi-bin/get-prodoc-entry?PDOC00867.

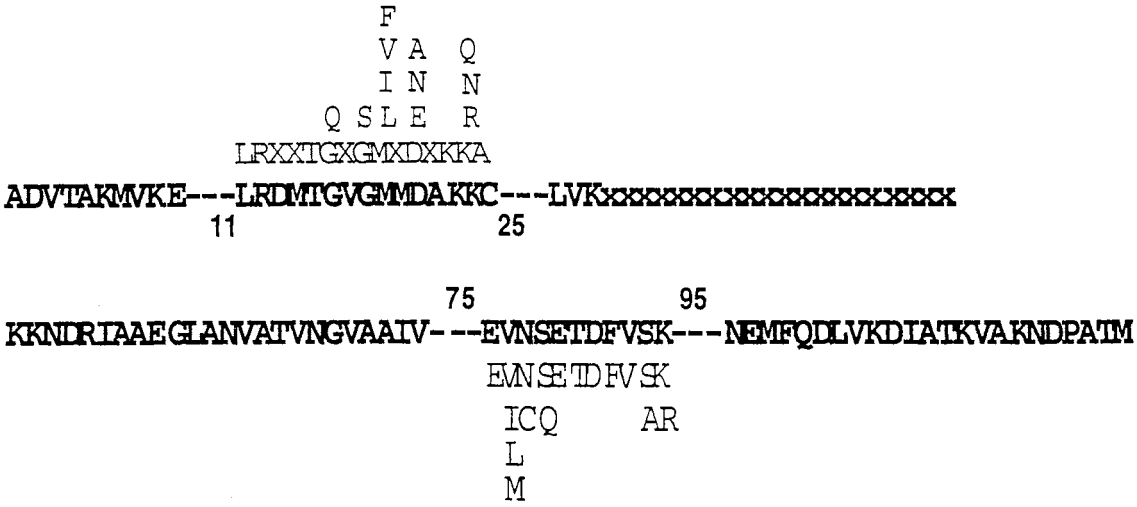

Figure III-14. Alignment of Band I and Band III with EF-Ts Signature Sequences. Amino acids in bold letters represent the band I and band III sequence. Dashes in the sequence set off the signature sequence from the rest of the sequence. Numbers indicate the first and last amino acid in the band I and band III sequences which match the signature. Plain type letters represent the signature sequences while letters in a vertical line indicate acceptable variations at the given position.

A BLAST+BEAUTY [BLAST (65)] search with the 30 amino acids of band II did not provide a positive identity. The best match was with an internal segment (amino acids 177-203) of a lipoamide dehydrogenase from *Mycobacterium leprea* with 48% identity and 62% similarity across the 30 residues. A Blitz search through the European Molecular Biology Laboratory server (Blitz@EBI.AC.UK) gave the best match against the first 29 amino acids of the UDP-Glucose 6-Dehydrogenase from *Streptococcus pyogenes* with 12 matches (40% identity), 7 mismatches, 9 partial matches (70% similarity) and one gap across the 30 residues. A BEAUTY (66) search through the Human Genome Center, Baylor College of Medicine with xnu & seq filters gave the best match between amino acid 12-27 of band II and amino acid 46-61 of endo-1,3-beta-glucosidase from *Hordeum vulgare* with 62% identity and 68% similarity across the 15 residues. A BEAUTY search with no filters gave the best match between amino acid 2-19 of band II and amino acids 7-24 of lactate dehydrogenase from *Bacillus subtilis* with 44% identity and 69% similarity across the 18 residues. Alignment of the Band II sequences with these proteins is seen in Fig. III-15.

UDP-glucose dehydrogenase is characterized by conserved motifs (67). One of these motifs is in the N-terminus and fits all eleven rules which define a NAD^+-binding fingerprint as defined by Wierenga (68). The NAD^+-binding fingerprint is defined by 11 residues spread across 30-32 amino acids which specify a "βαβ fold" that binds the adenosine moiety of NAD^+. Comparison of the band II sequence against the eleven rules is shown in Fig. III-16. HTP band II comforms to all ten of the rules which occur in the span of sequence that we have. Therefore, I conclude that HTP band II is a protein that contains a NAD^+-binding region.

```
UDP-GLUC DEHY  1 MKIAVAGSGYVGLSLGVLLSL-QNEVTIVD 29
                 MKIA+AG+G +G    G++L    +N+V ++
band II        1 MKIAIAGAGAMGSRFGLMLKQGRNDVLLII 30


GLUCOSIDASE       --------46 GSGIGLILDVGGNDVL 61--
                             GS  GL+L  G NDVL
band II        1 MKIAIAGAGAMGSRFGLMLKQGRNDVLLII 30


band II                      18 MLKQGRNDVLLII 30
                                ++ QG  D L++I
LAC DEHY     ---7 KVALIGAGFVGSSYAFALINQGITDELVVI 36-----
                 K+A+ GAG +GS +   L
band II        2 KIAIAGAGAMGSRFGLMLKQGRNDVLLII  30


LIP DEHY     ----177 IVIVGAGAIGIEFGYVLKNYGVDVTIV 203-----
                      I I GAGA+G  FG +LK    DV ++
band II        1 MKIAIAGAGAMGSRFGLMLKQGRNDVLLII 30
```

Figure III-15. Alignment of Band II Amino Acid Sequence. UDP-GLUC DEHY is UDP-Glucose Dehydrogenase from *Streptococcus pyogenes* (A46089). GLUCOSIDASE is endo-1,3-beta glucosidase from *Hordeum vulgare* (P34742). LAC DEHY is lactate dehydrogenase from *Bacillus subtillis* (P13714). LIP DEHY is lipoamide dehydrogenase from *Mycobacterium leprea* (699253). Two alignments are indicated with LAC DEHY. Letters between the two sequences designate identity, + designates similarity and blanks designate mismatch.

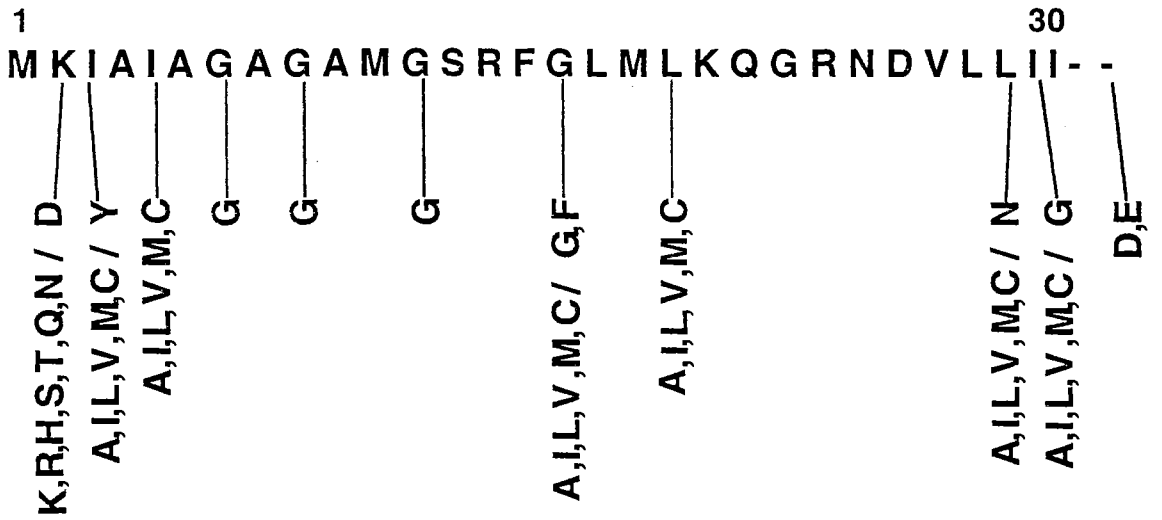

Figure III-16. Analysis of HTP Band II Against Wierengas Rules for NAD⁺ Binding Amino Acid Fingerprints. The horizontal sequence is the sequence of HTP band II and the vertical list of amino acids are the amino acids which must occur at the designated position in order to define an NAD⁺-binding motif. The amino acids following the / in the rules are observed exceptions.

Published Lipoamidase Sequences

The only lipoamidase sequence in the databases, (accession # gi 299494) is from human breast milk (EC 3.1.1.13). The 33 amino acids shown below were determined from an endoproteinase Lys-C generated fragment (46). Analysis showed the enzyme to actually be pancreatic cholesterol esterase.

Human breast milk lipoamidase (pancreatic cholesterol esterase)

(KIPFAAPTKA LENPQPHPGW QTLK)(KLVSEF TIT)

Interestingly, the part of the 33 amino acids beginning KIP and ending TLK [offset by ()] aligns with amino acid 53-78 of the cholesterol esterase, but does not contain two G residues, and the part beginning KLV aligns with amino acid 369-377. Band I, II and III do not align with the 745 amino acid protein.

A search of The Institute for Genomic Research (TIGR) database with the band I, II and III sequences revealed that the genes from *E. faecalis* which code for these proteins

have in part been sequenced. All of the band II sequence is found in the translation of the gene gef_6215. Part of band III is found in the TIGR gene gef_6365. The band I sequence is not found.

To separate further the 3 bands obtained from HTP1 and HTP2 more HTP columns were run using the LA-*p*ABA assay to probe protein peaks. HTP3 (Fig. III-17) gave a chromatogram that was similar to HTP2. The flow-through peaks were less pronounced than with HTP2 and there were more small peaks early in the elution. The peak at approximately fraction 23 looks like the HTP2 fraction 18 peak but it is at ~220 mM sodium phosphate and has a larger trailing shoulder. The bump at approximately fraction 35 is at about the same sodium phosphate concentration as in HTP2. The activity profile from HTP3 shows the major LA-*p*ABA cleaving activity in the ~220 mM sodium phosphate peak with some at ~100 mM and even less at ~400 mM sodium phosphate. No SDS-PAGE analysis was done from HTP3 as the A_{280} pattern and activity profile did not indicate much difference from HTP2

In HTP3 there was an obvious trailing shoulder on the major protein and LA-*p*ABA peak. To separate this further, HTP4 (Fig. III-18) was run with an altered gradient profile. The gradient was increased from 10 to 150 mM sodium phosphate in 5 min, then to 325 mM over 30 min, and finally increased to 500 mM over 10 min instead of the 25 min 10 to 500 mM sodium phosphate gradient used previously. At first glance the chromatogram looks similar to HTP3 except that the broad flow-through and early elution peaks are combined and are more pronounced and the bump at ~400 mM is flattened. The altered gradient did not result in a clean separation of the ~220 mM peak from the trailing shoulder but it did separate two peaks of LA-*p*ABA hydrolysis activity. The LA-*p*ABA peaks are separated by one fraction. LipDom inactivation assays were performed on fractions 33 and 40 to verify lipoamidase activity in the LA-*p*ABA active fractions. Fig. III-19 shows this data.

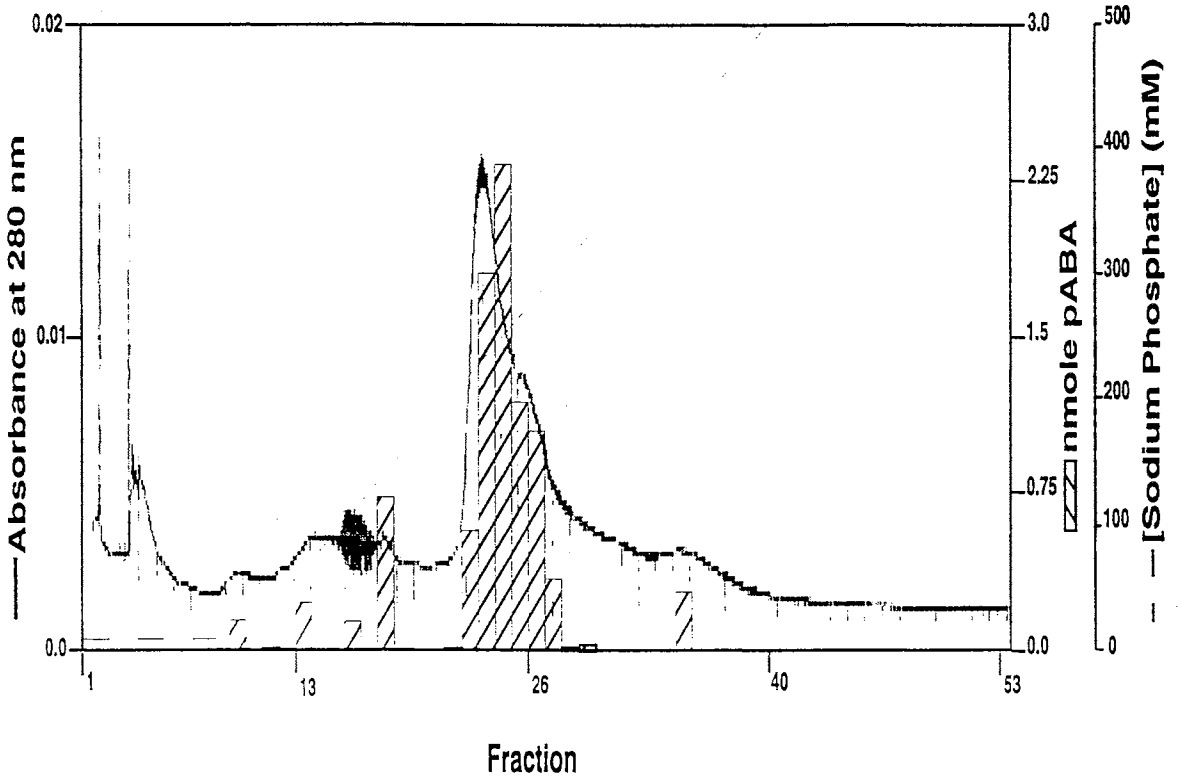

Figure III-17. HTP3 Hydroxylapatite Chromatography. Hatched bars indicate LA-*p*ABA hydrolysis activity. Dashed line indicates sodium phosphate elution gradient. Only the fractions indicated were assayed.

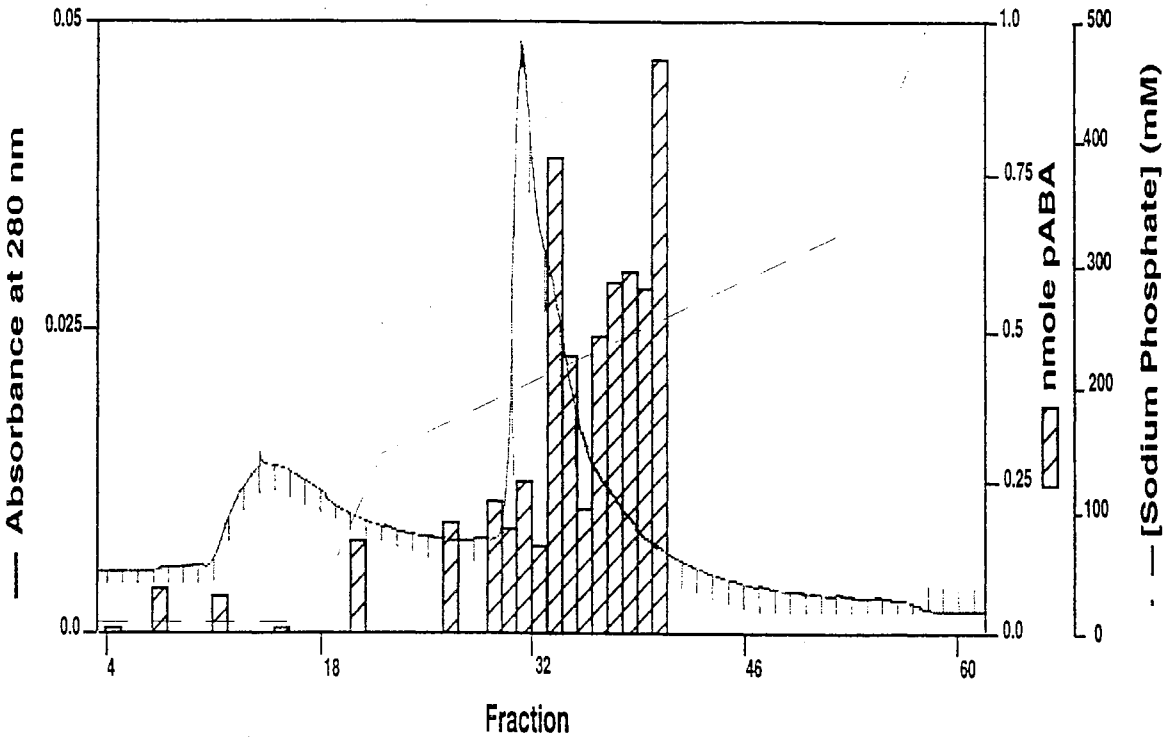

Figure III-18. HTP4 Hydroxylapatite Chromatography. Hatched bars indicate LA-*p*ABA activity. Dashed line indicates sodium phosphate elution gradient. Only the fractions indicated were assayed. Fraction #33 and #40 were assayed for LipDom inactivation (Fig. III-19).

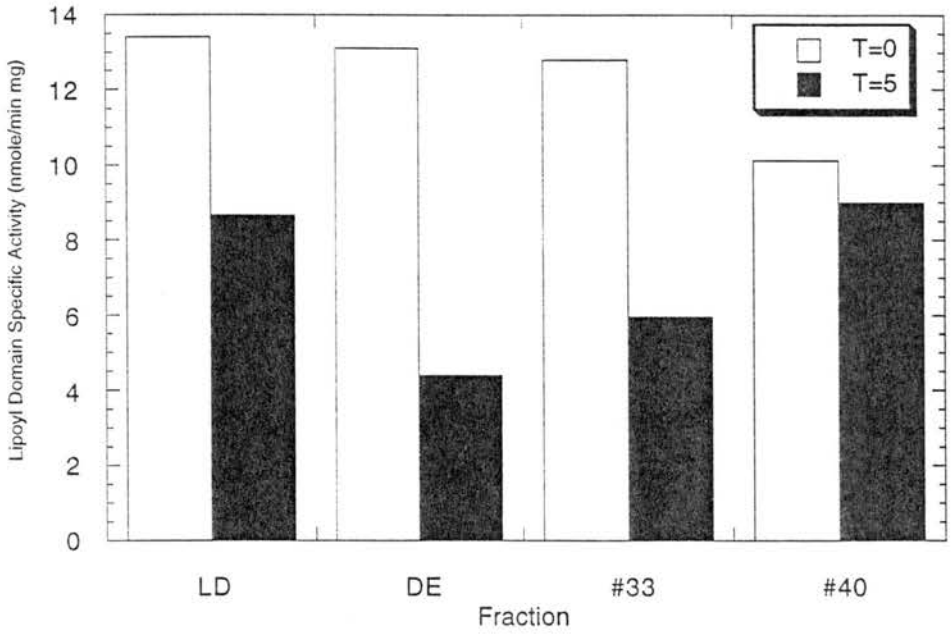

Figure III-19. LipDom Inactivation Activity in HTP4 Fractions. LD is lipoyl domain only +control, DE is the DEAE enzyme preparation which was loaded onto the column, #33 and #40 are HTP fraction 33 and 40 respectively. Incubation time was 5 h at 37 °C. Fraction #33 LipDom inactivation is significant at P=.05 with a t-stat of 6.32, Fraction #40 is not significant with a t-stat of 1.87. T-Critical at P=.05 is 2.9.

Due to the 4.7 nmole/min·mg decrease in lipoyl domain activity with the LD control and since the LD set, DE set and #33 set all had nearly identical T=0 activities, and the LD and #40 set had nearly identical T=5 activities, the T=5 activities were analyzed by a two sample z-Test to determine if the average difference between the treatments was significant. DE, #33 and #40 were all compared to LD control at p=.05. The DEAE purified preparation reduced the LipDom activity a statistically significant 4.2 nmole/min•mg, fraction 33 reduced the lipoyl domain specific activity by a statistically significant 2.7 nmole/min•mg and fraction #40 had no effect. Again I found that not all fractions exhibiting LA-*p*ABA cleaving activity remove lipoic acid from the lipoyl domain. Fraction #33 was eluted at ~ 200 mM sodium phosphate; fraction #40 was eluted at ~250 mM.

Based on the HTP4 results another HTP column (HTP5) was loaded and run under the same conditions as HTP4 and an identical chromatogram was obtained. The fraction which corresponded to fraction 33 from HTP4 was concentrated and the buffer changed to 20 mM potassium phosphate pH 7 in a spin ultrafiltration device. It was then electrophoresed on an 8% native polyacrylamide gel. Protein bands were sliced and assayed for LipDom inactivation. A portion of each band was saved and SDS-PAGE profiles were obtained (Fig. III-28). No LipDom inactivation was observed in the crushed gel pieces. However a native PAGE profile of the HTP fraction and SDS-PAGE profiles of the Native slices were obtained but did not give any further insight into lipoamidase identity.

E. Size Exclusion Chromatography

Size exclusion chromatography was performed on a TSK 3000 SW column with AS3DP fraction to determine what resolution could be obtained and whether lipoamidase activity could be detected in the fractions. All fractions assayed across the elution peak produced a significant loss of PDH activity; thus there was not sufficient resolution of proteins in the range of interest to make the approach profitable.

F. Hydrophobic Interaction Chromatography

HIC was tried in an attempt to utilize potential protein hydrophobicity for separation. There are two ways in which lipoamidase might react with a hydrophobic column. The first is the obvious and most common, the interaction between hydrophobic areas on the surface of the protein and the column. The second would utilize the natural substrate requirement of lipoamidase for the eight carbon lipoic acid where lipoamidase might bind the column in the active site.

A butyl and an octyl column were tried under different loading and elution conditions but no lipoamidase activity and very little protein was retained on the columns. The method was abandoned.

G. CM Cation Exchange Chromatography

An Econo CM column from Bio-Rad was used with the dialyzed pellet from the third ammonium sulfate cut (AS3DP). No lipoamidase activity was retained on the column so the method was abandoned.

H. MonoQ Anion Exchange Chromatography

Since anion exchange chromatography purified lipoamidase, a resin other than DEAE was examined. Initial experiments were done with a Bio-Rad Econo Q (quaternary amine) column run in Bis-Tris buffer and eluted with a linear gradient of 0.0-1.0 M NaCl in Bis-Tris, pH 7.0. This column yielded a small amount of protein washed through and a peak in the eluant. Analysis of some of the fractions with the PDH inactivation assay showed a peak of activity in the washed through protein and in the NaCl eluant but it is difficult to separate the activity in the eluant from the effect of NaCl on PDH. An analysis of the fractions with the LA-*p*ABA assay also found activity in the washed through protein and in the NaCl eluant. Verification of lipoamidase activity by inactivation and reactivation of PDH was unsuccessful. SDS-PAGE showed that none of the active fractions were homogeneous. Based on these results a MonoQ FPLC column was tested in order to get better resolution and more activity.

A MonoQ HR5/5 column loaded with pooled active fractions from the 0.15 M KCl DEAE peak gave little protein in the flow through while the eluant gave an interesting pattern from about 0.125 M NaCl to approximately 0.375 M NaCl with at least 10 peaks all run together (Fig III-20). Initial assay of the fractions with the LA-*p*ABA assay found

strong activity (Fig. III-21) in the flow through fraction (160 mmole/min/mg) and much weaker activity in the eluant (2.4 nmole/min/mg). A check of the most active LA-*p*ABA fractions with the LipDom inactivation assay showed no activity in flow through fraction 8 but strong LipDom inactivation in the eluant fraction 52 (p=.05) (Fig. III-22). SDS-PAGE was performed on a group of fractions around fraction 52. A ~ 36 kDa band which seemed to correlate in intensity with peak LA-*p*ABA cleavage activity was selected and N-terminal sequence determined. A sequence for 28 amino acids was obtained and the first 9 amino acids were the same as band I obtained from HTP chromatography (Fig. III-11).

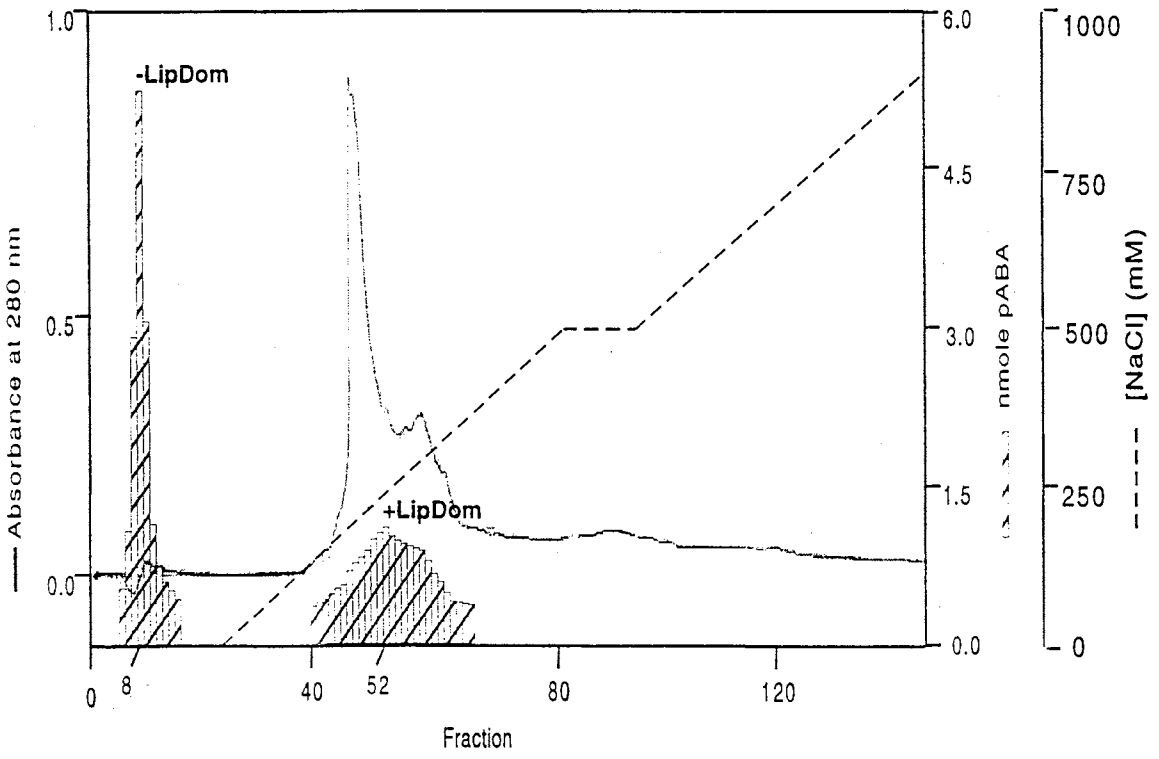

Figure III-20. Q1 MonoQ Chromatography with 0.15 M KCl DEAE Purified Lipoamidase Loaded. Hatched bars indicate LA-*p*ABA hydrolysis activity. Dashed line indicates KCl elution gradient. Fraction 8 and 52 were assayed for LipDom inactivation. Fraction 8 reduced LipDom activity a statistically non-significant 2.5 nmole/min•mg (t-stat = 2.4), fraction 52 reduced LipDom activity a statistically significant 17.5 nmole/min•mg (t-stat = 23.8, t-Critical for P=.05 is 2.9).

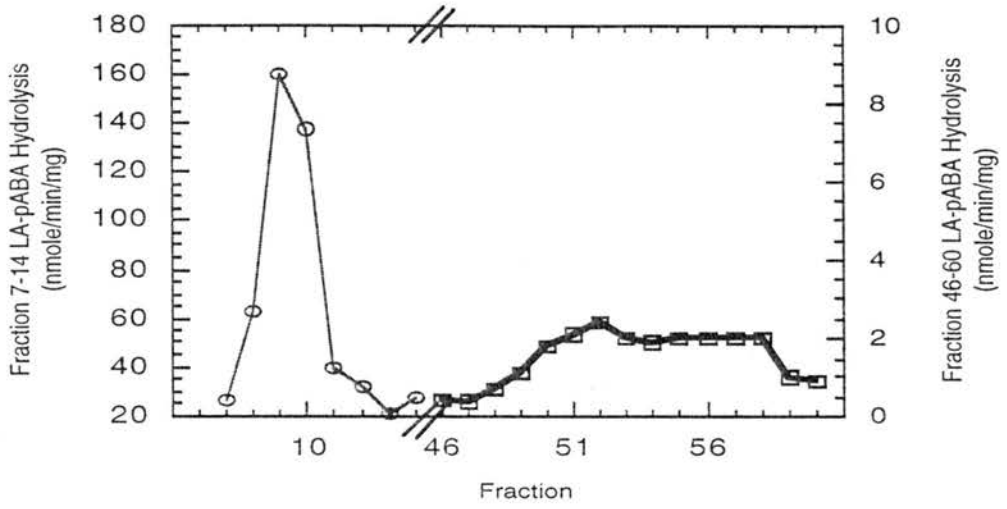

Figure III-21. LA-pABA Activity in MonoQ Fractions. Circles are the flow through fractions. Boxes are KCl eluant fractions. Note the difference in scale, the peak activity in the flow through is 160 and KCl eluant is 2.38 nmole/min/mg.

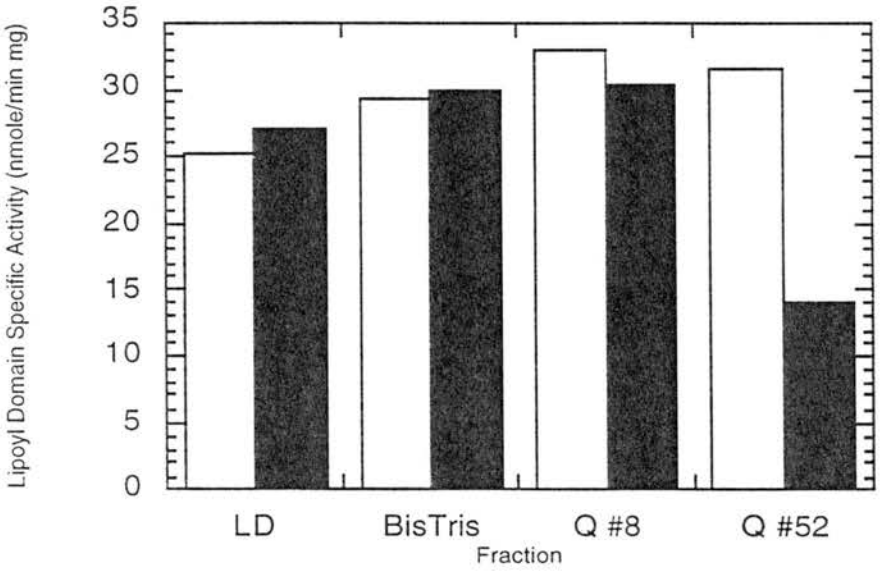

Figure III-22. LipDom Inactivation Activity in MonoQ Fractions. Outlined bars are T=0 values and solid bars are values after 4 h incubation with the indicated fraction. LD is the lipoyl domain only positive control, BisTris is the MonoQ running buffer negative control, Q #8 is fraction number 8 from the MonoQ column (t-stat = 2.4) and Q #52 is fraction 52 from the MonoQ column (t-stat = 23.8, P =.05 t-Critical is 2.9).

Three other MonoQ columns were run with the 0.15 M DEAE fractions loaded and reproducible chromatograms and activity profiles were obtained. One DEAE column run yielded LipDom inactivation activity in both the 0.1 M KCl (lipoamidase activity of 408 nmole/min/mg in the peak fraction) and 0.15 M KCl elution (lipoamidase activity of 117 nmole/min/mg in the peak fraction). The active fractions from these peaks were pooled separately and run on the MonoQ column with the result of nearly identical chromatograms and activity profiles (Fig. III-23). Even when HTP active fractions were loaded the profiles were nearly identical to DEAE loaded MonoQ runs and purification was only slightly better (Table III-4).

Table III-4. Purification of Lipoamidase On MonoQ Based on the LA-*p*ABA Assay. Activity data is based on the generation and detection of *p*ABA from LA-*p*ABA cleavage.

Fraction	Specific Activity (nmole/min·mg)	Total Activity (nmole/min)	Yield %	Purification
0.15 M DEAE	0.95	2.36		
Q #52	2.33	0.09	3.8	2.5
0.15 M DEAE	0.95	2.36		
HTP	3.25	0.10	4.2	3.4
Q #50	5.30	0.03	1.0	5.6

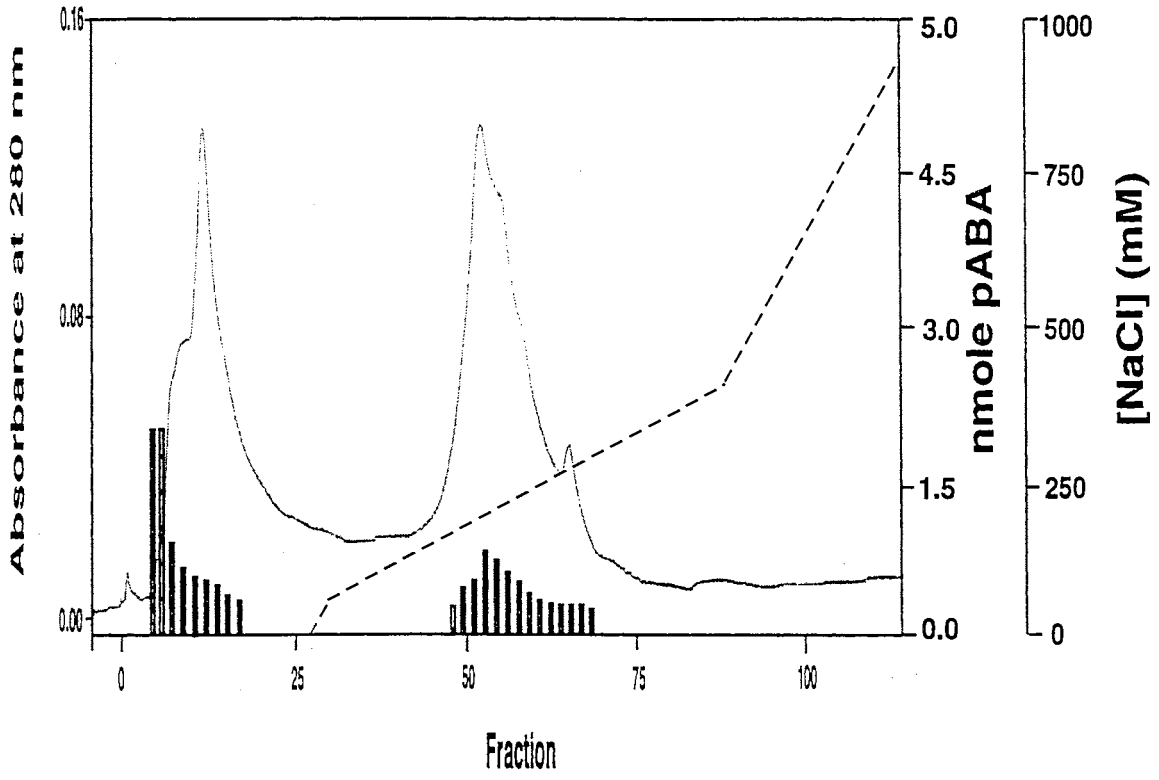

Figure III-23. MonoQ Anion Exchange Chromatography. This figure shows 0.1 M KCl DEAE purified lipoamidase. The 0.15 M KCl DEAE purified fractions from the same column had nearly identical activity but the activity of the flow-through peak is reduced. Hatched boxes indicate LA-*p*ABA hydrolysis activity and dashed line indicates NaCl elution gradient.

I. Attempt to Purify Lipoamidase from E. coli

An attempt was made to purify lipoamidase from *E. coli* (Crookes Strain) using the same procedure as for *E. faecalis*. The protamine sulfate step worked very well for removal of PDH from the extract but no lipoamidase activity could be detected. Koike and Suzuki (69) also reported no lipoamidase activity in this strain. Thus I verified their observations.

J. Summary of Additional Purification

Table III-5 lists purification data for HTP and MonoQ fractions starting from DEAE active fractions. DEAE active fractions were the final step in the Reed, Suzuki protocol (8) where the PDH inactivation assay was used. The HTP fractions assayed with the PDH inactivation assay (Table III-5) can be compared to their results and constitute further purification of the enzyme lipoamidase. Table III-2 shows that starting material (pH 6 SONS) has a specific activity of 9.6 nmole/min·mg and 1136 nmole/min total activity (data not shown in table III-2). From this beginning material HTP fraction II27-29 shows 801 fold purification with 5.4% yield. However the activity in HTP II27-29 was not verifiable by the PDH inactivation / reactivation assay.

Table III-5. Summary of Additional Lipoamidase Purification Beyond DEAE. * values reported are decrease in nmole acetylphosphate generated/min·mg; ^values reported are nmole TNB lost /min·mg; ` values reported nmole *p*ABA generated/min·mg. Notes; 1- these are the fractions sequenced. ; 2- From an HTP column run under slightly different conditions from that which produced I24-26 etc. ; 3- this fraction was also assayed by LipDom Inactivation. ; 4- this fraction was also assayed by LA-*p*ABA cleavage.

Method	Fraction	Starting Material	Assay Used	Specific Activity	Purification	Recovery	Notes
HTP		0.15 M DEAE	PDH Inactivate	*553.8			
	I23-25			*2210	4.0	7.8%	1
	II24-26			*2100	3.8	2.1%	1
	II27-29			*7714	13.9	5.4%	1
HTP		0.15 M DEAE	LipDom Inactivate	^97.1			
	HTP #33			^141	1.46	60%	2
MonoQ		0.15 M DEAE	LA-*p*ABA	`0.95			
	Q #52			`2.33	2.5	3.8%	3
MonoQ		0.1 M DEAE	LA-*p*ABA	`5.68			
	Q #42			`347	61	3.7%	
MonoQ		0.15 M DEAE	LipDom Inactivate	^117			
	Q #52			^721	6.2	76%	4

K. Protein Profiles from Verified Lipoamidase Fractions

Five fractions verified to have lipoamidase activity by either a recoverable PDH inactivation or by LipDom inactivation were analyzed by SDS-PAGE and silver staining. Comparison of the silver stained SDS-PAGE profiles of these fractions shows many protein bands ranging from ~27 kDa to ~65 kDa (Fig. III-24). All five fractions have a

band at 30-31 kDa, one at 33-34 kDa, one at 35-36 kDa and one at 38-40 kDa. Table III-6 shows the occurence of these bands and their relative quantity.

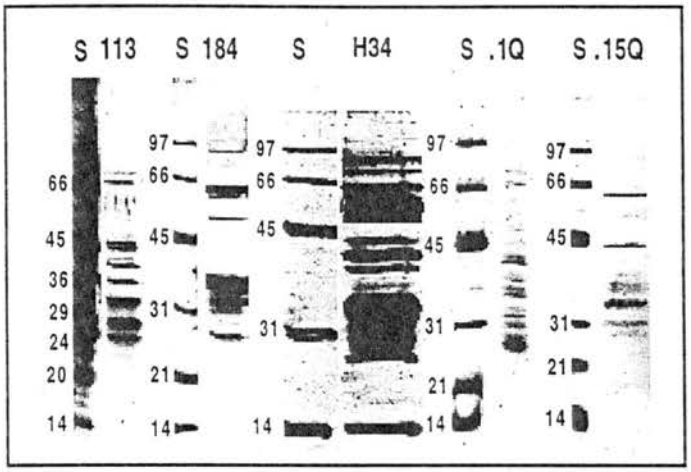

Figure III-24. SDS-PAGE Banding Profile of Fractions with Either PDH Inactivation/Reactivation or LipDom Inactivation Activity. S= molecular weight standards. 113= DEAE 0.1 M KCl fraction. 184=DEAE 0.15 M KCl fraction. H34= hydroxylapatite fraction. The .1Q= monoQ fraction from 0.1 M KCl DEAE load. 15Q= monoQ fraction from 0.15 M KCl DEAE load. All lanes are silver stained images. All S lanes are from the same gel as the fraction they are paired with.

Table III-6. Composition of Total Stained Protein Bands in the 30-40 kDA Range from Lipoamidase Fractions. Band percentages were determined by densitometry using an Alpha Innotech ChemImager 4000. Values reported are the % of band density the particular band contibutes to band density of all bands in the lane (for example the 31 kDa band from the CHT sample comprises 14% of total protein stained in the CHT sample).

Band MW (kDa)	% of CHT bands	% of 0.15 DE>Q bands	% of 0.1 DE>Q bands	% of 0.1 DE bands	% of 0.15 DE bands
30	-	-	07	17	02
31	14	07	10	05	22
32	-	-	07	-	-
33	20	-	08	15	16
34	-	08	06	-	-
35	-	21	09	02	21
36	08	-	-	-	-
37	-	06	-	-	-
38	-	03	-	11	-
39	04	03	-	-	02
40	-	-	04	04	-

CHAPTER IV

DISCUSSION

A. General

Lipoamidase first came to my attention when I began a project to examine lipoic acid addition to and removal from multi-enzyme complexes. There are four systems in which lipoic acid activation has been studied: animal, plant, yeast and bacteria. Research was underway in other laboratories on the animal system and *E. coli*. This left the yeast system to be examined further. To study lipoic acid activation a substrate, (a non-lipoylated) α-keto acid dehydrogenase, is required. One way to acquire this substrate is by treating holo dehydrogenase with lipoamidase. This is why I originally started out to purify lipoamidase, but it is clear that lipoamidase is a very useful tool.

Lipoic acid is attached to the ε-NH_2 of lysine residues, and the disulfide to sulfhydryl redox reaction is essential for α-keto acid dehydrogenase function. Lipoic acid is also involved in recognition of phosphorylation sites in α-keto acid dehydrogenases by regulatory kinases. Lipoic acid (lipoyl domains) also occur in proteins other than the α-keto acid dehydrogenases. However the function of lipoic acid in these other proteins is not clear. There are two ways to study lipoic acid involvement and mechanism: mutagenesis of the lipoic acid containing protein to remove the lipoylation site or specific removal of the lipoic acid from purified protein. The result of either approach is a protein

with either partial or complete deletion of lipoic acid. Observing the effect of delipoylation on activity/kinetics of the protein gives insight into the function of lipoyl domains. Of the two approaches the application of lipoamidase is the easiest and most straightforward.

Lipoylation/delipoylation is also possibly a control system used to modulate activity of lipoyl containing enzymes similar to adenylation/deadenylation with glutamine synthetase, but involves a functional cofactor. Glutamine synthetase sensitivity to feedback inhibition is altered by the degree of adenylation on tyrosine residues; the higher the degree of adenylation the more susceptible the enzyme is to product inhibition (70). Recall from Chapter I that lipoic acid is involved in interactions with a regulatory kinase in eucaryotic α-keto acid dehydrogenases (16). Also recall that in *E. coli* approximately half of the lipoyl domains can be removed with no apparent overall decrease in dehydrogenase activity (18). The degree of lipoylation may alter dehydrogenase responsiveness to product feedback inhibition or phosphorylation/dephosphorylation. Lipoamidase could be involved in regulating the degree of lipoylation and it would be useful in studying whether such regulatory mechanisms exist.

Recently Dr. Thomas Roche at Kansas State University expressed an interest in lipoamidase as a tool for his studies on the role of lipoic acid in the phosphorylation/dephosphorylation of the α-keto acid dehydrogenases. Personal communication with him revealed that his lab had worked on purifying lipoamidase for a time but had abandoned that pursuit. Dr. Roche graciously provided recombinant human lipoyl domain and an assay (LipDom inactivation assay) for determining the lipoic acid content of those domains to be used as an assay for lipoamidase activity.

Many papers have been published concerning mammalian lipoamidase but no complete sequence information has been published. The only known sequence is a partial amino acid sequence from human breast milk (chapter III p. 76). There are some discrepancies involving enzymes assumed to be lipoamidase. In serum and breast milk, enzymes assumed to be lipoamidase are in fact biotinidase (43, 44) and a form of pancreatic

cholesterol esterase (46) respectively. Oizumi (47) found that the pig brain enzyme called lipoamidase is a multiple hydrolase since the only specificity requirement is that lipoic acid be the acyl group of the substrate.

Much remains unknown about lipoamidase and it is not presently conveniently available as a research tool. To fill this need I set out to purify lipoamidase to homogeneity, determine a partial amino acid sequence (either N-terminal or internal), design oligonucleotide probes based on the protein sequence and then use the probes to clone and sequence the lipoamidase gene. Once the gene is cloned it can be used in an expression vector which will allow production and purification of large quantities of active lipoamidase.

B. Initial Experiments

I began to isolate lipoamidase by following the purification procedure of Suzuki and Reed (31) since it (see Fig. II-6) so effectively and easily separates the PDH, lipoamidase and the activating system.

Early experiments were encouraging, after the DEAE step there was only one major band (~36 kDa) and 3-5 minor bands (>35 kDa) present in the active fractions by Coomassie Blue staining of SDS-PAGE gels (Fig. III-1). I was able to inactivate holoPDH and then reactivate it by incubation with lipoic acid and an activating system preparation (Fig. III-3). Table III-2 shows however that purification was 58 fold with 10 % yield. Since the objective was to obtain homogeneous lipoamidase, I needed to eliminate the other proteins.

C. Further Purification with Hydroxylapatite Chromatography

Initial experiments with hydroxylapatite (HTP) column chromatography gave encouraging results in that only 3 protein bands were seen in the active fractions. Fig. III-8

shows activity data for 6 fractions. Initially the DEAE preparation and HTP fractions 9-10, and 22-23 were assayed. When fraction 23 was found to have activity, fractions 24 and 25 and the elution buffer were assayed. Notice that the elution buffer alone (Fig. III-8, "Buffer") inactivated PDH as much as fraction 23 but it was only half of what fractions 24 and 25 had inactivated. As previously noted the only way to be certain that observed PDH inactivation is due to lipoamidase and not protease or high salt or other factors is to reattach lipoic acid to E2 and observe PDH reactivation. The inactivation/reactivation assay was attempted with these fractions but was not successful. Unfortunately all of my activating system preparations after early 1994 were unable to reactivate inactivated PDH and they also reduced the activity of control PDH even though they were capable of activating *E. faecalis* apoPDH. When reactivation failed, fraction 22, 23 and the elution buffer were reassayed with 3 times as much sample. Fraction 23 showed ~ 3 fold increase in PDH inactivation while fraction 22 and the elution buffer showed no increase. This indicated that the activity in fraction 23 was not simply due to a salt effect. SDS-PAGE was performed on the fractions from HTP1 and fractions from the next run (HTP2). Fig III-10 shows three bands were present at ~32 kDa, 35 kDa and 37 kDa in all three samples. Examining the relative signal intensity (Table III-3) of the three bands from the three pooled samples and the PDH inactivation specific activity of the pooled samples suggests that band I is lipoamidase. Specific activity is greatest in HTP2 27-29, lower in HTP1 23-25 and lowest in HTP2 24-26. Band I signal intensity is also greatest in HTP2 27-29 and lowest in HTP2 24-26. Band II signal intensity is greatest in HTP2 24-26 and lowest in HTP1 23-25 and band III is roughly equal in the three fractions. Since HTP2 27-29 has the greatest specific activity and there is relatively more band I in this fraction than the other two fractions band I may be lipoamidase.

These three bands were sequenced and analyzed. The amino acid sequence similarity between band I and band III with EF-Ts and the presence of an EF-Ts signature sequences in both band I and band III (Fig. III-13, III-14) indicate that these proteins are

EF-Ts. EF-Ts is part of the translation machinery in prokaryotes where it binds the EF-Tu GDP complex and induces the exchange of GDP for GTP which primes the elongation complex for the next round of amino acid addition. EF-Ts remains bound until GTP has been hydrolyzed. EF-Ts is also present in eucaryotic chloroplasts (72) and mitochondria (73) where its function is presumed to be similar.

Band I contains the N-terminus of EF-Ts and may be the complete protein since its molecular weight is similar to other bacterial EF-Ts proteins. Band III corresponds to a fragment of EF-Ts beginning at amino acid 51. Fig. III-12 shows the alignment of the band I and band III sequences together against 4 other bacterial EF-Ts sequences. Examination of the TIGR data base revealed that at least part of the genes for the band I, II and III proteins have been sequenced. The *E. faecaslis* sequences found in the TIGR database are raw DNA sequencing data which are classified as "Enterococcus faecalis unfinished fragment of complete genome" and have not been published. The TIGR gene gef_6365 contains the coding sequence for amino acid 7- 58 of band III with a one base pair deletion which creates a framshift in the putative protein. Band I is not found but the first 6 amino acids of band III is not found either. This suggests that the N-terminus of the EF-Ts gene has not been sequenced by TIGR. There is little doubt that the band I and band III proteins are EF-Ts. The sequence of *E. faecalis* EF-Ts has not been reported and I have determined 86 of the putative 109 amino acids. I will submit this to one of the databases by direct submission as a putative EF-Ts.

The partial EF-Ts sequence from *S. pneumoniae* was determined by Kolberg *et al.* (78) by screening bacterial and mammalian cellular protein extracts with antibodies raised against pneumococcal cell extracts. One of the antibodies reacted with a ~43 kDa protein. The N-terminal sequence of 28 amino acids identified this protein as EF-Ts. The antibody reacted with all streptococcal strains tested (12 total) but neither of the two enterococcal strains (including *E. faecalis*). The antibody did not react with any of the other bacterial strains or mammalian cells tested. The authors state that differences in reactivity with this

antibody between streptococcal and enterococcal strains support the taxonomic split of streptococci and enterococci into separate genera. If band I is the full length protein, *E. faecalis* EF-Ts has a molecular weight of 36-37 kDa. *S. pneumoniae* is ~43 kDa; this is further support for the new classification. Of the 5 proteins examined the *S. pneumoniae* is the largest at ~43 kDa, the other 4 are all smaller with *E. faecalis* band I at 36-37 kDa, *S. citrii* at 32.8 kDa, *E. coli* at 30.2 kDa and *H. influenzae* at 30.0 kDa.

Analysis of HTP band II shows it to be similar to the amino terminus of UDP-glucose dehydrogenase from *S. pyogenes* (a Gram+ cocci) and other dehydrogenases (Fig. III-16). But 40% identity and 70% similarity across only 29 amino acids is not an absolute identification. Examination of the TIGR database showed that the gene encoding the band II has been partially sequenced and identified as gef_6215. The evidence that band II is a dehydrogenase is strengthened by the identification of a NAD^+-binding fingerprint in the same location as the one present in the UDP-glucose dehydrogenase.

Even with the identification of band I and band III as EF-Ts they cannot be eliminated as being lipoamidase. An SDS-PAGE protein band from a single MonoQ column fraction ,#52, which had LA-*p*ABA hydrolysis and LipDom inactivation activity is EF-Ts. The fraction was not homogeneous by SDS-PAGE but the band was selected because it was a major band, it was present only in fractions 51-53 and it correlated with LA-*p*ABA activity in the fractions. Note that all of the fractions in the peak were assayed with the LA-*p*ABA assay and fraction 52 was assayed with the LipDom inactivation assay. Thirty amino acids of the sequence were obtained from the band, and the first nine were identical to the HTP band I (EF-Ts). This finding, along with the HTP results, places EF-Ts in fractions which are active with all three assays. Finding EF-Ts in fractions with activity for all three assays is not proof that EF-Ts is lipoamidase, but the correlation between EF-Ts signal intensity and PDH inactivation specific activity in the sequenced fractions is further evidence that it is.

EF-Ts is not just a binding protein which regulates EF-Tu during protein biosynthesis. Highly purified EF-Ts preparations from *E. coli* also have guanylate cyclase activity (74). Also the EF-Tu.Ts complex is part of the RNA bacteriophage Q β RNA replicase (75). The EF-Tu•Ts complex is required for iniation of RNA synthesis (75) but EF-Ts does not dissociate from EF-Tu in the process as it does in its normal function (76).

There are also examples of lipoamidase with multiple activities and other enzymes with lipoamidase activity. Amino acid sequencing of LA-*p*ABA cleaving lipoamidase in human milk identified it as bile salt-stimulated lipase. Mutagenic abolition of the esterase activity did not affect the LA-*p*ABA activity (46). This is a lipoamidase enzyme with two independent activities. The enzyme in pig brain which cleaves lipoyl-lysine has been characterized as a multiple hydrolase (47, 48) which will cleave amide, ester, or peptide bonds. There is no data as to the independence of these activities but it is not a rigidly specific enzyme.

There are many other proteins which have multiple activities. Adenylosuccinate lyase (ASL) catalyzes two reactions in the purine synthetic pathway, but in *Bacillus subtilis* it also has a role in regulating glutamyl-tRNA synthetase activity (71). These two activities by the same protein may be a control link between nucleotide and protein synthesis. Mammalian acetyl-CoA carboxylase, which is involved with fatty acid biosynthesis, catalyzes a CO_2 activation reaction and a carboxylation. Both active sites and a biotin carboxyl carrier are contained in a single polypeptide (70). Mammalian fatty acid synthase is 500 kDa homodimer which catalyzes seven different reactions in fatty acid synthesis from acetyl-CoA and malonyl-CoA (70).

Acetyl-CoA carboxylase and fatty acid synthase catalyze sequential reactions in a biosynthetic pathway, but one of the activities of *B. subtilis* ASL appears to be unrelated to the other activities. The regulation of glutamyl-tRNA synthetase is a binding function from a protein which also catalyzes the elimination of fumarate from adenylosuccinate. If EF-Ts is also lipoamidase it follows the same pattern; a single protein with both catalytic

(lipoamidase) and regulatory binding (EF-Ts) activity. Although the connection is not evident, it is possible that EF-Ts is also lipoamidase and may provide a regulatory link between carbohydrate metabolism (and thereby cellular metabolic condition) and translation.

The physiological function of lipoamidase is unknown. Its capacity to remove lipoic acid from lipoyl containing proteins suggests two possible functions. One function might be in regulation of α-keto acid dehydrogenase complexes since removal of lipoic acid results in diminished or abolished complex activity. Recall that Stepp *et al.*(18) found that with the *E. coli* α-keto acid dehydrogenase about half of the lipoyl groups in the complex could be removed without significantly affecting complex activity. This makes the complex activity very stable but after removal of half of the lipoyl groups complex activity would be very sensitive to lipoamidase removal of lipoyl groups. The other function could be lipoic acid salvage and recycling. Oizumi (38) found that human serum lipoamidase could remove lipoic acid from bovine PDH only after the PDH had been proteolyzed. Neither function would require a stand alone enzyme and a single protein with both EF-Ts and lipoamidase activity would not hamper either activity.

Since lipoamidase was not pure and the correlation between EF-Ts and lipoamidase was not absolutely solid, more HTP columns were run. These were done using the LA-*p*-ABA assay and altering the elution gradient. No further separation of the three bands was achieved, but lipoamidase activity was established by the LipDom inactivation assay in a fraction corresponding to the ones sequenced. This fraction has multiple bands by silver staining. Three of the twelve bands are the same molecular weight as the three bands sequenced (Fig III-28 lane H34, and Table III-5). The presence of proteins of about the same size as those sequenced in a fraction which inactivates LipDom is further reason not to dismiss EF-Ts as being responsible for lipoamidase activity. For the same reason neither can I dismiss band II even though it is not evident why an amide hydrolase would need an NAD^+-binding domain. Also, finding LipDom inactivation activity in a fraction

corresponding to the ones from which the three sequences were obtained establishes the presence of lipoamidase in the sequenced fraction in the absence of PDH reactivation.

D. Further Purification by MonoQ Ion Exchange Chromatography

Since I was not able to purify lipoamidase to homogeneity with hydroxylapatite chromatography, I examined MonoQ ion exchange chromatography. The first column run (Fig. III-20) was loaded with active DEAE purified sample and had at least 10 A_{280} peaks run together. Fractions were screened with the LA-*p*ABA assay and two peaks of activity were identified, one very active (fraction 8 peak) which did not inactivate lipoyl domains and one very weak (fraction 52 peak) which inactivated lipoyl domain by half (30 nmole/min·mg to 15 nmole/min·mg). Table III-4 shows that fraction 52 achieved only 2.5 fold purification of LA-*p*ABA activity with just 3.8% yield from the previous step. Table III-5 shows 6.2 fold purification and 76% yield of LipDom inactivation activity from the previous step. This large difference in yield between the LA-*p*ABA assay and the LipDom inactivation assay indicates how non-specific the LA-*p*ABA assay is for lipoamidase. Yet it is still useful for screening large numbers of fractions. When fraction 52 was analyzed by SDS-PAGE 6 protein bands were found (Fig. III-28 .15Q lane and Table III-5). The most prominent band (~36 kDa), was only found in fractions 51-53 and was sequenced and identified as EF-Ts. As previously discussed this is the same protein which was sequenced in 2 bands from hydroxylapatite chromatography.

With lipoamidase activity found in MonoQ fractions from 6 silver stained proteins I attempted to further separate the 10 peaks and 6 proteins by altering the elution gradient and the sample loaded on the column. Extending the gradient did begin to separate the A_{280} peaks but it also diluted LA-*p*ABA activity to undetectable levels. Changing the fraction loaded onto the MonoQ column to hydroxylapatite, or 0.1 M KCl DEAE purified sample did not result in homogeneous protein. Table III-4 shows purification data for MonoQ

fractions with DEAE purified protein loaded and with DEAE>HTP purified protein loaded. The purification with HTP sample is only 5.6 fold with only 1% yield.

E. Multiple Peaks of LA-pABA Activity

The presence of multiple peaks of LA-*p*ABA cleaving activity from column chromatography was a recurring event. It first occurred with DEAE fractions using the LA-*p*ABA cleavage assay. Two peaks of activity were detected with the most active one unable to inactivate PDH. In later purifications, the sample which was applied to HTP and MonoQ columns was from the DEAE peak which inactivated PDH. Subsequently multiple LA-*p*ABA activity peaks were also found with MonoQ fractions (DEAE>monoQ and DEAE>HTP>MonoQ). MonoQ fractions were assayed with the LipDom inactivation assay to establish lipoamidase activity. Again I found that the peak with greatest LA-*p*ABA activity did not inactivate lipoyl domains. It may be that there are multiple enzymes, which can cleave LA-*p*ABA in *E. faecalis,* which are being separated on these columns. The lipoamidase literature supports this possibility (see below). In fact in rat liver there are two pH optima for LA-*p*ABA hydrolysis but only one optimum for lipoyl-lysine hydrolysis (42). Thermostability, pH stability, inhibition pattern, tissue abundance and subcellular localization data indicate that the enzyme responsible for lipoyl-lysine hydrolysis activity is different from LA-*p*ABA hydrolysis but the same as biotinidase. It is also possible that partially degraded lipoamidase can hydrolyze the synthetic LA-*p*ABA substrate but not the natural substrate.

With mammalian lipoamidase investigations, enzymes having lipoamidase activity as defined by LA-*p*ABA hydrolysis were subsequently found to be other enzymes or have loose specificities. Human serum LA-*p*ABA and lipoyl-lysine hydrolyzing activities respond differently to inhibitors (41). These two activities are different enzymes and the one responsible for lipoyl-lysine cleavage is, in fact, biotinidase (42). Garganta *et al.* (43)

compared human serum biotinidase and lipoamidase characteristics and immunoprecipitated both activities with an anti-biotinidase antibody. They concluded that the two activities were due to the same enzyme. Nilsson *et al.* (44) concluded that serum lipoamidase activity was biotinidase when a boy with severe biotinidase deficiency was also lipoamidase deficient. Serum lipoamidase and biotinidase activities copurified through six steps (45). In human breast milk the enzyme referred to as lipoamidase was purified, proteolytically cleaved and partially sequenced. It is a bile-salt stimulated lipase instead of lipoamidase (46). Oizumi (47, 48) found the enzyme from porcine brain to require lipoic acid in its substrate but in an amide, ester, or peptide bond. There were only loose requirements for lipoic acid attachment, they referred to this enzyme as a multiple hydrolase. These cases set a precedent for using synthetic substrates for the determination of lipoamidase activity but many of them provide evidence that synthetic substrates are not very specific.

Table IV-1 lists enzymes which were originally identified as lipoamidase. Both the human bile salt stimulated esterase (pancreatic cholesterol esterase EC 3.1.1.3) and human biotinidase (EC 3.5.1.12) have been cloned and sequenced. There are no detectable identical regions between the two yet both have LA-*p*ABA cleaving activity. The only two proteins originally designated lipoamidase which still are, are liver enzymes. The enzyme from rat liver though has two pH optimums, this indicates either 2 active sites in one enzyme or multiple enzymes. Chromatography data generated during this purification of *E. faecalis* lipoamidase suggests there are multiple LA-*p*ABA cleaving enzymes in this system also. In all instances only one of the fractions could inactivate PDH or LipDom which indicates that one of the LA-*p*ABA cleaving fractions is not really lipoamidase.

Table IV-1. Lipoamidase and Biotinidase Comparisons. Initial Designation is what the enzyme was first identified to be, Current Designation is what it is now called after further experiments. Liplys is lipoyl-lysine, ser hydrolase designates a serine type hydrolase.

Initial Designation	Weight (kDa)	Activity Measured	Comments	Current Designation
Lipoamidase	60-70	LA-*p*ABA, esterase		Cholesterol esterase
Lipoamidase	51	LA-*p*ABA, esterase	Rat clone, 2 active sites	Cholesterol esterase
Biotinidase	61	B-*p*ABA	Human clone	Biotinidase
Biotinidase	66-76	B-*p*ABA	Human	Biotinidase
Serum lipoamidase	76, 110	B-*p*ABA, Liplys, LA-*p*ABA	Human, 20k fold enriched	Biotinidase
Liver biotinidase	70	B-*p*ABA	Guinea pig, thiol hydrolase	Biotinidase
Liver lipoamidase	60	LA-*p*ABA	Guinea pig, ser hydrolase	Lipoamidase
Brain lipoamidase	140	Liplys	Pig, 600 fold enriched	Multiple Hydrolase
Breast milk lipoamidase	135	LA-*p*ABA, lipolytic	4.4k fold enriched, ser hydrolase	Cholesterol esterase
Liver lipoamidase		Liplys, B-*p*ABA	Rat	Biotinidase
Liver lipoamidase		LA-*p*ABA	Rat, 2 optimal pH	Lipoamidase

All of the above instances are from mammalian sources and so I ask, what about prokaryotic lipoamidase? The only report of prokaryotic lipoamidase is the groundbreaking work of the Lester Reed group (29, 31) which is the basis for this attempt to purify lipoamidase from *E. faecalis*. Suzuki and Reed indicated that post DEAE *E. faecalis* lipoamidase removed protein bound lipoic acid and hydrolyzed methyl lipoate, lipoamide,

and lipoyl-lysine as well as other lipoyl amino acids but not biotinyl-lysine, acetyl-lysine or benzoyl-lysine.

The LA-*p*ABA assay is not an unequivocal indicator of lipoamidase activity but it is a fast, easy means of screening column fractions. The PDH inactivation/reactivation assay is the only assay that eliminates questions about lipoamidase activity. In early experiments (Fig. III-3) this assay was used to determine lipoamidase in DEAE fractions. I modified the assay by recovering inactivated PDH in a spin filtration device, rather than the standard pelleting of PDH at 144k × g, so that the assay was much more reproducible. But activating system preparations began to further reduce PDH activity instead of recovering it. I never resolved this problem; therefore, I lost the ability to establish that PDH inactivation was due to lipoamidase removal of lipoic acid instead of something else like dissociation of the complex, or protease digestion of the complex. Even in attempts to purify the heat and acid stable PS2B protein of the activating system I had trouble with activation of apoPDH. Interestingly the activating system positive controls with the PDH inactivation/ reactivation assay always activated *E. faecalis* apoPDH. I have no explanation for this.

The LipDom inactivation assay was used in place of the PDH inactivation assay as a measurement of lipoic acid removal. This assay can be used after DEAE purification. Prior to the DEAE step there is a component that interferes with the analysis. Fig. IV-1 shows that mixing pre-DEAE sample with the LipDom inactivation assay components generates almost 8 times more TNB than the amount of lipoyl domain used in the assay. After DEAE purification none of this interfering activity remains and the assay is useful but I cannot use the LipDom inactivation assay throughout a purification scheme.

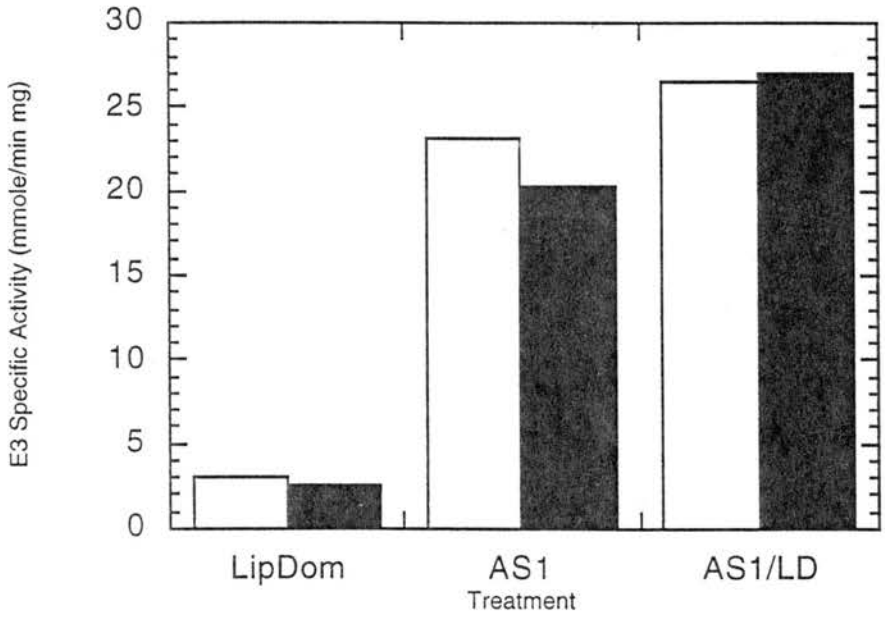

Figure IV-1. Interference with LipDom Inactivation Assay. White bars represent the average T=0 values and black bars represent the average T=3 hr values. LipDom indicates lipoyl domain incubated with buffer. AS1 indicates an ammonium sulfate lipoamidase preparation incubated with buffer. AS1/LD indicates lipoyl domain and the ammonium sulfate preparation mixed together.

I initially planned to examine the yeast activating system's ability to attach lipoic acid to mammalian E2 lipoyl domains. With the receipt of recombinant human lipoyl domains from Dr. Roche I was able to make a similar examination with lipoamidase and determine that *E. faecalis* lipoamidase can remove lipoic acid from human lipoyl domain resulting in the loss of E3/DTNB activity (Fig. II-5). This is interesting in light of Oizumi's findings (38) showing human serum lipoamidase is able to remove lipoic acid from bovine PDH only after the PDH had been trypsinized. However, Ravindran *et al.* (64) used partially purified *E. faecalis* lipoamidase to remove lipoic acid from both the complete PDH complex and recombinant domains in a study of the role of lipoyl moieties in kinase stimulation of mammalian PDH activity. This further indicates that not all enzymes called lipoamidase are the same.

F. Other Chromatographic Separation Methods Examined

All chromatography methods discussed in this thesis are based on ionic properties. These are the only separation methods that I found to work. Dye affinity chromatography was attempted using a test kit consisting of nine different dye conjugated columns from Sigma. The mode of binding with dye affinity chromatography is not well defined for any given protein and may well involve charge characteristics, affinity binding at the active site, regulatory sites or surface affinity. One of the columns showed some promise for retaining LA-*p*ABA cleavage activity during initial screening but the results could not be repeated.

Hydrophobic interaction chromatography (HIC) was explored using a Pharmacia Hi-Trap HIC test kit consisting of different types of C4 and C8 columns. A C4 and a C8 column were tested. The C8 column was selected in an attempt to mimic the 8 carbon basic structure of lipoic acid and attract the lipoamidase active site. Neither the C4 or the C8 column bound much protein and did not bind any LA-*p*ABA cleaving activity.

G. Native PAGE

No combination of chromatographic steps produced homogeneous lipoamidase. The HTP column yielded three protein bands in the active peak but I was not able to unequivocally associate one with lipoamidase. There is evidence that the EF-Ts 36-37 kDa band from the HTP fraction is lipoamidase. However two native PAGE experiments with DEAE purified lipoamidase gave a single SDS-PAGE band of ~36. One of these native PAGE experiments showed a slight reactivation of inactivated PDH (Fig. III-27) and the other showed strong LA-*p*ABA hydrolysis. An attempt to sequence this protein was unsuccessful.

These results show that native PAGE can resolve the DEAE purified proteins to single bands. For this reason a great deal of effort was spent trying to establish lipoamidase activity in these fractions. Native PAGE purified protein can be accurately assayed for by the LA-*p*ABA cleavage assay but we have already seen that the assay is prone to false positive results. The slight reactivation of PDH seen in the slice number 9 assay was the only increase in PDH activity seen with any of the gel slices. The recovery of 5% of a 95% inactivation may not seem very convincing but when all other slices showed a further inactivation of PDH, 5% becomes significant. Sequencing of this band was unsuccessful. The conditions were not worked out which would allow the LipDom inactivation assay to be used successfully with native gel slices.

H. Lipoamidase Molecular Weight Range Analysis

Since implementation of the lipoyl domain cleavage assay, all fractions from liquid chromatography were initially screened by the LA-*p*ABA assay and then the most active fractions were analyzed with the LipDom inactivation assay. Fractions from DEAE (both the 0.1 M and the 0.15 M KCl), MonoQ loaded with both 0.1 M KCl and 0.15 M KCl DEAE, and from CHT-II hydroxylapatite showing LA-*p*ABA and lipoyl domain inactivation activity were examined by SDS-PAGE. Figure III-28 shows that none of the fractions were homogeneous. It is tempting to compare the band profile versus molecular weight profiles of these fractions and say that since only the 31 kDa band is present in all of these fractions it must be lipoamidase. This approach does allow me to eliminate proteins outside of the 30-40 kDa range. Table III-6 compares the molecular weight and relative staining intensity as determined by densitometry of the 30-40 kDa proteins from the LipDom inactivation active fractions. From Table III-6 I could say that only the 31 kDa protein is present in all fractions but I am not comfortable saying that the protein determined to be 36 kDa in HTP is not the same protein determined to be 35 kDa in all of the other

fractions. While in theory one should be able to say that a given protein and a given set of molecular weight markers will migrate the same way every time, in practice I have found this is not necessarily true. I have run two different molecular weight markers (Bio-Rad and Sigma) on my gels whenever I have the room and have found them to migrate slightly differently relative to each other with every gel.

Interestingly though a protein of ~36 kDa has consistently been prominent in various experiments. One of the very first gels run with DEAE active fractions was Coomassie Blue stained and showed the major band to be ~35-36 kDa. Also the one native PAGE experiment which showed inactivation/reactivation of PDH in a gel slice had a single SDS band of ~36 kDa. One of the last native slice experiments done showed a strong LA-*p*ABA cleavage activity in a gel slice and a single SDS band of ~36 kDa. Band II from HTP, which was sequenced and found to be very similar to UDP-Glucose dehydrogenase, migrates at ~35 kDa. And the band from a LipDom inactivation active MonoQ fraction which was sequenced and identified as EF-Ts migrated at ~36 kDa on that gel, although the band from HTP which yielded the same sequence migrated at ~37-38 kDa. I can confidently conclude that lipoamidase is a protein of ~35-38 kDa molecular weight.

I. Further Purification

Purification calculations (Table III-2) show that lipoamidase was purified 58 fold through DEAE chromatography with a 10 % yield. These results were obtained using the PDH inactivation assay. Lipoamidase activity in a corresponding sample from a previous DEAE column was established by PDH inactivation/reactivation. Application of this sample to hydroxylapatite chromatography resulted in 801 fold purification and with less than 1 % yield by the PDH inactivation assay. The three protein bands in this fraction were sequenced. At this point reactivation of PDH was not possible due to unexplained failure of my activating system preparations. Further HTP columns and all MonoQ columns were

assayed with the LA-*p*ABA assay and the LipDom inactivation assay was then used to establish lipoamidase activity. These two assays do not allow calculation of purification starting from crude extract so data was calculated from the DEAE step (Table III-4). Recall that a component of the pre-DEAE fraction gives a very high background with the LipDom inactivation assay and the LA-*p*ABA assay gives false positives in fractions which do not have lipoamidase. None of the fractions with LipDom inactivation activity was purified more than 6 fold beyond DEAE but the yield was 60 % or greater. Compare this to the PDH inactivation assay used on HTP fraction II27-29 with ~14 fold purification and 5 % yield beyond DEAE purification. These levels of purification and recovery are not very good but they could be tolerated if the fractions contained only one or two protein bands. None of them do.

I did not purify lipoamidase to homogeneity. This may be difficult as it has not been accomplished in the laboratories of Dr. Lester Reed or Dr. Thomas Roche who are very skillful enzymologists. One difficulty is the lack of specificity using model compounds such as LA-*p*ABA as substrate. It is clear there are multiple proteins with hydrolytic activity toward LA-*p*ABA.

Whether the EF-Ts protein that was sequenced from column fractions which contain lipoamidase activity is really lipoamidase remains an unanswered question. EF-Ts has multiple functions including protein elongation, guanylate cyclase activity and initiation factor in the RNA phage Q β RNA replicase.

I did purify Elongation Factor Ts from *E. faecalis* and sequence 86 (amino acid 1- 28 and 51-108) amino acids. There is an apparent correlation with PDH inactivation in HTP fractions and EF-Ts. EF-Ts is present in MonoQ fractions which have LipDom inactivation activity. These sequences will be submitted to an appropriate database as a putative EF-Ts.

J. *Summary*

The goal of this project was to purify lipoamidase to homogeneity. An already established protocol was used initially and then with hydroxylapatite (HTP) chromatography, fractions were obtained which had been purified ~800 fold and contained three proteins. Sequencing of these three proteins identified two of them as EF-Ts. The other was unidentified but contains an NAD^+-binding domain in the amino terminus. Various types of chromatography were evaluated for purification but only HTP and anion exchange (MonoQ) purified lipoamidase. The three proteins from HTP were not further resolved nor significantly enriched. Analysis of band patterns from LipDom inactivation active fractions indicate that lipoamidase is a ~35-38 kDa protein. EF-Ts sequence was obtained from three different protein bands purified from active fractions in two chromatographic methods, 86 amino acids of the *E. faecalis* protein were determined. Lipoamidase may correlate with one of the sequenced EF-Ts bands from HTP chromatography.

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