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A Characterization of L-SD#1, the Gene Product of *sdaA* in *Escherichia coli* K-12

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ABSTRACT

A Characterization of L-SD#1, the Gene Product of *sdaA* in *Escherichia coli* K-12

John Moniakis, Oct. 1992

L-Serine deaminase #1 (L-SD#1) from *E. coli*, synthesized in an inactive form that requires activation *in vitro* and *in vivo*, was purified in 2 part fusion (2PF) and 3 part fusion (3PF) β-galactosidase fusion protein forms, which demonstrate possible conformational differences between one another.

The 3PF protein contained a collagen sequence linking the L-SD#1 and β-galactosidase protein molecules, whose digestion using collagenase to yield separate β-galactosidase and L-SD#1 protein molecules was demonstrated. Purified protein was used to raise antibodies to 2PF protein, which were used for identifying L-SD#1 in bacterial extracts.

This work proved that L-SD#1 (48.8 kDa) is indeed coded for by *sdaA*. It was also revealed that the translational start site of *sdaA* was 18 bp or 6 amino acids upstream of what was previously thought.

An analysis of L-SD#1 activation and activity *in vitro* revealed the Km for activated enzyme as being 25.5 mM for L-serine and found glycine to be an inhibitor of L-SD#1 activity.

Two forms of inactive L-SD#1 may exist, IN#1 and IN#2, which can be activated with low and high amounts of DTT respectively. The activation mechanism of L-SD#1 had been theorized to involve a proteolytic cleavage, for which no evidence was found in this investigation, but evidence suggesting a conformational change was revealed.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I INTRODUCTION

L-SD (L-Serine Deaminase) is an Escherichia coli K-12 enzyme that breaks down L-serine to pyruvate, ammonia, and water. The enzyme has been characterized in other bacterial species. L-SD is involved in the conversion of L-serine to pyruvate in the uric acid fermentation pathway of Clostridium acidi-urici [Benziman et al., 1960]. This enzyme requires a highly reducing environment, with complete inhibition by sulfhydryl binding reagents suggesting that protein thiols are vital for activity, and iron for activity in vitro [Benziman et al., 1960]. In Diplococcus glycinophilus [Klein and Sagers, 1961] and Athrobacter globiformis [Bridgeland and Jones, 1965] L-SD is necessary for the organisms to grow on glycine as the carbon source. In vitro these enzymes do not require the addition of a cation but do demonstrate a substrate induced activation [Gannon et al., 1976]. In Escherichia coli K-12, the enzyme is produced in a considerable amount [Pardee and Prestidge, 1955], but serves no obvious purpose [Newman et al., 1985b].

In Escherichia coli K-12 the enzyme L-SD, both in vivo and in vitro, is obtained in an inactive form. From studies on E. coli strains (MEW128, MEW191 and MEW84) that lack in vivo activity but have an activatable enzyme in vitro, the inactive form is thought to be activated in vivo enzymatically and in vitro chemically (with iron and dithiothreitol (DTT)) [Newman et al., 1985b; Newman et al., 1985a].

In this thesis I present a study on the characterization of E. coli L-SD both in vitro and in vivo. The work presented in this thesis is a continuation from previous work in which the L-SD gene (sdaA) had been cloned and fused to the β-galactosidase gene (lacZ) in two different forms, the 2 part fusion (2PF) and the 3 part fusion (3PF) proteins. The fusion proteins were constructed for purification purposes, i.e. L-SD is covalently bound to β-galactosidase and can thus be co-purified with β-galactosidase using a β-galactosidase affinity column [Su, 1991].

The preliminary work presented in this thesis quantifies the purification of L-SD fusion proteins. This revealed that the two fusion proteins (2PF and 3PF) may have different conformations. Purified protein was used to verify the sdaA gene sequence by N-terminal amino acid sequencing.

The purified protein was used in the characterization of L-SD in vitro. Polyclonal antibodies were raised in rabbits, for the purpose of identifying L-SD in E. coli extracts.

For the purpose of performing a kinetic investigation, a coupled LDH/NADH continuous assay was modified and used for monitoring L-SD activity. Using this, the specific activity and Km of purified L-SD were determined.

L-SD was studied in its inactive form and found to be activatable with both β-mercaptoethanol (ME) and DTT. It was also revealed that inactive L-SD can assume a second inactive form that can only be activated with DTT at a 10 times higher

level than under normal activation conditions.

Iron and DTT are known to cleave proteins [Kim et al., 1985], and so it was postulated that the activation of L-SD involved a cleavage, possibly at a serine-serine bond [Su et al., 1989]. The in vitro activation process was studied and no evidence for a cleavage involved in or even accompanying L-SD activation was observed. However, some evidence suggests that a conformational change may be involved in activation.

The in vivo activation of L-SD was also investigated. When in the fusion protein form, L-SD showed no activity in vivo, even though no loss of activity was found in vitro (after activation with iron and DTT), suggesting that the attached β-galactosidase may prevent activation in vivo. L-SD activation in cells was studied with the idea that protein folding may be involved, and indeed data revealed that chaperone proteins (proteins that assist in the proper folding of their protein substrates in vivo) may play a role in L-SD activation.

In order to provide a background to the investigation presented in this thesis, the following parts of the introduction will review the experimental evidence related to enzyme activation and L-SD function.

PART 1. *IN VITRO* ACTIVATION OF L-SD WITH IRON AND DTT

Most of the enzymology presented in this thesis was done *in vitro* with iron and DTT as the activating agents of L-SD. In this part of the introduction, I will review what is known about L-SD activation along with what is known about the action of iron and DTT on proteins.

1-1. *In Vitro* Activation of L-SD

In extracts, L-SD is obtained in an inactive form that requires activation. L-SD is activated *in vitro* by DTT, oxygen and either ferric (Fe III) or ferrous ion (Fe II). Even with ferrous ion, DTT is required, and with ferric ion oxygen is required. Indeed, activation did not occur at all in the absence of O_2 [Newman *et al.*, 1990]. This suggests that neither Fe II or Fe III is the activating species. Data also indicated that chelators stabilizing the oxidized form of iron inhibited L-SD activation. Taken together, these results suggested that activation involved a cycling of iron between the ferric and ferrous states, generating an active species in a reaction with oxygen in the process. One possibility is that the active species is a hydroxyl radical, as judged by hydroxyl radical scavenger experiments [Newman *et al.*, 1990]. In these experiments hydroxyl radical scavengers such as mannitol, alpha-naphthol, 8-hydroxyquinoline and L-histidine

inhibited activation when added during L-SD activation, but had little or no effect when added after L-SD had been incubated with Fe and DTT for 15 minutes.

Hydroxyl radicals and many other reactive species are formed when iron is cycled between ferric and ferrous forms. The ferrous iron (either added as Fe (II) or formed by the reduction of Fe (III)) is oxidized by molecular oxygen resulting in a superoxide radical anion, which would then undergo a dismutation reaction to form hydrogen peroxide. Hydrogen peroxide can then oxidize ferrous iron (Fenton reaction) in the process generating hydroxyl radicals [Walling, 1975]. Due to the possibility of iron cycling, whenever I refer to "iron (Fe)" throughout the thesis, I imply a mixture of both Fe (III) and Fe (II), as well as the other reactive species formed.

1-2. Hydroxyl Radical Characteristics and their Overall Effect on Proteins

Hydroxyl radical species are known to cleave proteins into lower molecular weight species. Low levels of cleavage were noticeable after one hour of incubation of glutamine synthetase with iron and DTT, with increasing proteolysis as the incubation time was increased [Kim et al., 1985]. The oxidative inactivation of carbamoyl phosphate synthetase was thought to involve cleavage through a hydroxyl radical species

[Alonso et al., 1992]. The metal-catalyzed oxidation of superoxide dismutase, involving hydroxyl radical species, cleaved the protein via a proposed complicated mechanism [Stadtman and Oliver, 1991].

Free radicals are highly reactive, and because of their high reactivity they have very short half-lives [Basaga, 1990], less than one nanosecond [Slater, 1984]. The L-SD activating species generated by iron and DTT is also short-lived but apparently much less so, 80% of the total activity is lost if Fe and DTT are pre-incubated for 30 minutes prior to the addition of enzyme [Newman et al., 1990]. Therefore, the stability for the activating species appears to be in the order of minutes. The highly reactive hydroxyl radicals often abstract carbon-bound hydrogen atoms non-selectively [Schuchmann and Von Sonntag, 1977] or add to carbon-carbon double bonds [Steenken and O'Neill, 1978]. Indeed, investigation of hydroxyl radical action on glutamine synthetase and glucose-6-phosphate dehydrogenase suggested that oxidative modification of critical amino acids in itself, and not protein cleavage, was the major mode of action of hydroxyl radicals on these proteins [Kim et al., 1985; Szweda and Stadtman, 1992].

It has also been reported that hydroxyl radical reactions may cross link polypeptides. Investigators observed the formation of higher molecular weight protein species upon incubation of proteins with iron and DTT [Penzes et al., 1984; Kim et al., 1985].

1-3. A Proposed Free Radical Activation Mechanism of L-SD

Knowing that hydroxyl radicals are involved in proteolysis it was proposed that activation of L-SD might involve a cleavage by Fe-DTT generated hydroxyl radicals liberating an active fragment(s) [Newman et al., 1990]. A possible site of proteolysis was thought to be the serine-serine bond of amino acids 243 and 244 [Su et al., 1989] by analogy with the enzyme histidine decarboxylase which is activated by serinolysis at a serine-serine bond [Resci et al., 1983]. However, the histidine decarboxylase activation reaction is intramolecular and does not involve either iron or DTT. Indeed, since iron and DTT are known to destroy proteins it seems odd that they would be involved in the activation of L-SD. If serinolysis is involved it may be a random process, iron and DTT cutting L-SD in many places with only a fraction of the cuts resulting in a serine-serine cleavage.

1-4. The Activation of Histidine Decarboxylase

Amino acid decarboxylases generally require pyridoxyl-5'-phosphate (PLP) as a cofactor [Gallagher et al., 1989]. Histidine decarboxylase (HDC) from Lactobacillus utilises the covalently bound cofactor pyruvate [Riley and Snell, 1963]. The enzyme is in an originally inactive form (π), which is then activated by a cleavage to yield two separate subunits,

α and β. Cleavage of the π chain occurs by serinolysis at the ser81-ser82 site, with the serine residue at the N-terminal of the α subunit being converted to a pyruvoyl group, and a serine-OH remaining at the C-terminal of the β-chain. The oxygen incorporated into the C-terminal of the β chain comes from the OH-group of the pyruvoyl-forming serine residue of the α-chain [Resci et al., 1983]. The two subunits remain associated and the active site is formed at the serinolysis cut site. The pyruvoyl residue is thought to facilitate decarboxylation via a Schiff base mechanism resembling that of the PLP dependent decarboxylases [Van Poelje and Snell, 1990]. By analogy, a proteolysis at the serine-serine bond of L-SD might result in a similar activation process, with the production of a pyruvoyl residue that could be involved in the L-serine deaminating reaction.

1-5. The Possible Individual Roles that Fe and DTT May Play in Enzyme Function

In addition to forming radical species that may cleave proteins, iron and DTT can also act separately on proteins and thus may be needed each in their own way rather than together as generators of an active species. DTT and ME are the favourite disulfide reducing agents in biochemical experimentation [Jocelyn, 1987]. DTT is a stronger reducing agent than ME because it forms a cyclic six-member disulfide

ring upon oxidation; the intramolecular reaction is faster than the corresponding intermolecular reaction for ME. Additionally, a balance is reflected between the thermodynamic stability of the CSSC dihedral angle and the entropy of formation of the ring from the acyclic reduced form [Burnes and Whitesides, 1990]. DTT completely reduces noncyclic disulfides to thiols, and intermediate mixed disulfides are not present in a significant concentration [Singh and Whitesides, 1991]. Therefore the activation process may involve DTT (and possibly ME as well) reducing disulfide bonds in L-SD.

1-6. Examples of Thiol and Metal Requiring Enzymes

Thiols are required at the active site in the case of tryptophan indole-lyase, where cys-298 is located at the active site possibly in contact with a pyridoxyl cofactor [Philips and Gollnick, 1989]. Reduced cysteines are also required in the case of E. coli FNR protein [Spiro and Guest, 1988] where they may function as iron-sulfur centres [Trageser, et al., 1990]. FNR protein is a regulatory protein that activates the transcription of its target genes in response to oxygen limitation, and is believed to be active under anaerobic conditions (reduced cysteines) and inactive under aerobic conditions (oxidized cysteines) [Spiro and Guest, 1988]. L-SD may require iron binding at an iron-sulfur

centre as in the case of L-serine dehydratase from Lactobacillus fermentum which was found to bind Fe (II), activating the enzyme through a conformational change and through a possible interaction with enzyme bound PLP [Farias et al., 1991].

There are other examples of metal binding and thiol requiring enzymes. E. coli L-threonine dehydrogenase is a tetrameric protein with 6 cysteines/subunit. Catalytic activity is stimulated 5 to 10 fold by added Mn2+ or Cd2+. One cysteine residue in particular has been identified as the binding site of the Mn^{2+} and Cd^{2+} [Epperly and Dekker, 1991]. L-Serine dehydratase from Peptostreptococcus asaccharolyticus was also found to contain an Fe-S centre. This enzyme functions only under anaerobic conditions and exposure to oxygen results in the enzyme's inactivation. However, activity can be regained under anaerobic conditions upon the addition of ferrous ion [Grabowski and Buckel, 1991].

PART 2. A SECOND L-SD IN E. COLI, L-SD#2

If cysteines play an important role in L-SD then they may be conserved in a second L-serine deaminating enzyme found in E. coli K-12, L-SD#2. This section will review what is known about L-SD#2 (L-SD from sdaA studied in this thesis has been termed L-SD#1 [Su and Newman, 1991]).

2-1. Studies on sdaB and the Implications for Cysteines

A second gene sdaB (mapped at 61.0 minutes) was found to code for a second L-SD enzyme, L-SD#2, which is only synthesized in complex media [Su and Newman, 1991] and so is not a factor in any of the work presented in this thesis. L-SD#2 also requires iron and DTT for in vitro activity. A comparison of the two enzymes might therefore indicate the features involved in activation.

A comparison of the sdaA [Su et al., 1989] and sdaB [Shao, personal communication] amino acid sequences reveals that there is a conservation of cysteines. The sdaA gene codes for 9 cysteines in its polypeptide product and the sdaB gene codes for 10 cysteines. Of these, 8 are at conserved positions in the two proteins. This, along with the requirement of iron and DTT in the in vitro activation mechanism for both enzymes suggests a possible importance of disulfide bonds in enzyme activation.

2-2. Other Examples of Proteins with Cysteine Homology

A large group of eukaryotic membrane damaging toxic proteins secreted by gram-positive bacteria belonging to the genera Bacillus, Clostridium, Listeria and Streptococcus share similar characteristics with L-SD#1 and L-SD#2 in that they are activated by thiol-reducing agents [Geoffroy et al.,

1990]. The genes encoding for five of these toxins have been cloned and sequenced. Like L-SD#1 and L-SD#2, the toxins share greater than 50% homology between their amino acid sequences, but more interestingly they all contain a cysteine at a conserved position near the C-terminal. Investigators believe this cysteine to be essential for enzymatic activity [Geoffroy et al., 1990]. Of the 8 conserved cysteines between L-SD#1 and L-SD#2, 7 are near the C-terminal of the protein. Perhaps one or more of these are essential for L-SD activity.

PART 3. THE MECHANISM OF FORMATION OF DISULFIDE BONDS

In the previous section the possible importance of disulfide bonds in L-SD was introduced. Therefore their formation in vivo may play an important role in enzyme function and will thus be reviewed in this section.

3-1. In Vivo Disulfide Bond Formation

The mechanism of disulfide bond formation in vivo is unknown. The formation of proper disulfide bonds proceeds most efficiently in an environment with redox conditions that favour correct disulfides and disfavour incorrect ones, with environmental conditions promoting a reversible thiol/disulfide exchange [Pigiet and Schuster, 1986].

In E. coli protein disulfide formation is thought to be

catalyzed by small proteins (about 12 kDa in size, called thioredoxins. In E. coli, thioredoxin is believed to reside in a specialized compartment of the cell from subcellular fractionation studies that show thioredoxin, although a soluble protein, does not co-purify with known cytosolic proteins or with periplasmic markers [Gleason and Holmgren, 1988]. Thioredoxins, which are also found in eukaryotes, have active-site sequences similar to protein disulfide isomerases, which are believed to promote disulfide formation in eukaryotes [Lundstrom and Holmgren, 1990]. Thioredoxin may function by providing a microenvironment where disulfide bond formation can proceed efficiently at cellular pH values which are not conducive towards disulfide bond formation [Nilsson and Anderson, 1991]. Sulfhydryl groups are only reactive when ionized to thiolate [Houk and Whitesides, 1987] and the pKa of the cysteine thiol is 8.3 [Stryer, 1975]. Therefore at physiological pH values, which are between 7.5 and 7.9 [Padan and Schuldiner, 1986], uncatalyzed disulfide formation reactions are slow. However, thioredoxin has a reactive thiol group with a pKa of 6.7 and can thus initiate disulfide bond formation [Kallis and Holmgren, 1980].

In vivo L-SD activity may be conformationally dependent on proper disulfide bond formation, and in turn proper disulfide bond formation may only take place if the protein is folded so that cysteines are adjacent to one another. Both in vivo L-SD activity and protein folding will be reviewed in Part 4 of the Introduction.

PART 4. *IN VIVO* L-SD ACTIVITY AND ACTIVATION

The activation of L-SD *in vitro* is thought to mimic L-SD activation *in vivo* [Newman *et al.*, 1985a]. Therefore, I will review what is known about L-SD activation and activity *in vivo*, along with possible activation mechanisms.

4-1. L-SD in Wild Type *E. coli*

L-Serine can not be used as a sole carbon source to sustain wild type *E. coli*. However, if the growth media is supplemented with glycine and leucine, then wild type cells can grow on L-serine as the sole carbon source [Newman and Walker, 1982b]. These two amino acids induce *sdaA* expression [Pardee and Prestidge, 1955; Newman and Walker, 1982b]. For growth on L-serine as the sole carbon source, L-SD must convert L-serine to pyruvate, with the cells then using pyruvate as the carbon and energy source. Using growth on L-serine as a selection tool, many mutants of L-SD have been isolated by our laboratory.

4-2. *In Vivo* L-SD Activation Mutants

Strains MEW128 (*sda128*), MEW191 (*sda191*) [Newman *et al.*, 1985b], and MEW84 (*sda84*) [Su, 1991] are all deficient in *in vivo* L-SD activity. The mutations were mapped at 15-17 minutes, 86.5 minutes, and 55 minutes for the *sda128*, *sda191* [Dumont, 1985], and *sda84* [Feng, 1990] respectively. These mutants showed wild type *in vitro* L-SD activity [Newman *et al.*, 1985a]. Since the gene which encodes L-SD (*sdaA*) has been mapped at 41.0 minutes [Su *et al.*, 1989], the MEW128, MEW191 and MEW84 mutants must have a functional sdaA gene and the *sdaA* gene product L-SD must be synthesized in these mutants, but in an inactive form that requires activation by at least three proteins, the gene products of *sda128*, *sda191*, and *sda84*.

4-3. The Heat Shock Response of L-SD and Chaperone Proteins

In previous work, it was observed that subjecting cultures to heat shock (raising the temperature from 37°C to 42°C) induces L-SD activity *in vivo* [Newman *et al.*, 1982a]. The synthesis of the chaperones, such as GroEL and GroES, is constitutive but augmented at 42°C [Neidhardt *et al.*, 1987]. Molecular chaperones interact with many unfolded or partially denatured proteins, allowing these proteins to attain their proper conformation [Langer *et al.*, 1992].

There are two basic classes of heat shock proteins, the Hsp60 and Hsp70 families. Members of both these classes of molecular chaperones use the energy of ATP hydrolysis in their protein folding processes. The Hsp70s identified in E. coli are DnaK, DnaJ, and GrpE proteins [Echols, 1990]. The Hsp60s identified in E. coli are the GroEL and GroES proteins [Fayet et al., 1986].

The mechanism of action of molecular chaperones has been proposed as comprising a cascade of reactions. First, DnaK, and/or a DnaK/DnaJ protein complex interacts with a newly translated amino acid sequence emerging from a ribosome, assisting the newly synthesized polypeptide in obtaining an intermediate folding conformation. This folded complex is then transferred to a GroEL/ES complex, using GrpE as the coupling factor, where the polypeptide obtains its native conformation [Langer et al., 1992].

In E. coli, the grpE gene [Ang and Georgopolous, 1989], and the groES/EL genes have been found to be essential for viability. Therefore, mutations in these alleles must still retain activity at some level otherwise the cells will not be viable [Veilstra-Ryalls et al., 1991]. It is possible that the three in vivo L-SD activity deficient mutants (MEW128, MEW191 and MEW84) may be lacking in appropriate levels of functional molecular chaperones, or chaperone like proteins, thus effecting L-SD folding and activity. However, the level of chaperones may still be high enough to allow for cell growth.

There are many examples of chaperone involvement in polypeptide folding. Chaperones have been found to be involved in bacteriophage lambda, T4, T5, and 186 assembly [Zeilstra-Ryalls et al., 1991], ß-lactamase membrane translocation [Bochkareva, et al., 1988], prokaryotic rubisco folding [Goloubinoff et al., 1989], mammalian citrate synthase folding [Buchner et al., 1991], and mammalian rhodanese folding [Langer et al., 1992].

4-4. In Vivo Activation of L-SD in a Fusion Protein Form

H. Su observed that the L-SD in vivo activating system did not appear to function on L-SD fused to β-galactosidase [Su, 1991]. For purification purposes sdaA-lacZ (codes for the 2 part fusion, 2PF, protein) and sdaA-proα-lacZ (codes for the 3 part fusion, 3PF, protein) fusions were constructed [Su, 1991]. The proα of the 3PF encoded the triple helical region of the chicken collagen gene. Thus treating purified 3PF with collagenase would liberate L-SD from ß-galactosidase [Germino and Bastia, 1984]. These fusion constructs showed no in vivo L-SD activity but showed high levels of in vitro activity after activation. It is not difficult to imagine that an enzymatic activation system designed to function on L-SD can not do so when L-SD is part of a bulky fusion protein molecule. However, small molecules like Fe and DTT might still find their way to an appropriate site.

PART 5. PURIFICATION AND PROPERTIES OF β-GALACTOSIDASE FUSION PROTEINS

The purification of the 2PF and 3PF proteins was a crucial part of this thesis not only for the purpose of obtaining purified protein but also in terms of observing possible conformational differences between the two protein species. This section will therefore review what is known about β-galactosidase fusion proteins and their purification.

5-1. History of β-Galactosidase Fusion Proteins

β-Galactosidase fusion proteins have been used as a tool in biochemistry/molecular biology for some time due to the simple assay for β-galactosidase and its stability. A β-galactosidase-insulin A or B chains [Goeddel et al., 1979] fusion protein construct is one of the earliest examples of such a fusion. In this case, the hormone amino acid sequence was fused to the C-terminal of β-galactosidase which had 17 amino acid residues deleted. These fusion constructs were insoluble and had only trace β-galactosidase activity. In contrast, a NH_2 terminus β-galactosidase fusion construct, lacI-lacZ gene product, had both repressor and β-galactosidase activities [Brake et al., 1978].

5-2. Structure and Properties of β-Galactosidase N-Terminal Fusion Proteins

Structural studies on fusion proteins revealed that up to 14 amino acid residues of β-galactosidase N-terminal can be substituted by a foreign sequence without any effect on the stability or activity of the hybrid. These fusions retained their tetrameric (wild type β-galactosidase forms a tetramer) form and had β-galactosidase activity equivalent to that of the wild type enzyme [Fowler and Zabin, 1983]. However, hybrids with up to 26 amino acids of the β-galactosidase sequence deleted dissociate into dimers more readily, and deletions of more than 26 amino acids resulted in a dimeric form of the protein with no β-galactosidase activity [Fowler and Zabin, 1983]. In the fusion proteins, the shape of the β-galactosidase polypeptide immediately following the foreign sequence was affected by the substitution, with the result that the overall conformation of the tetrameric fusion protein was found to be more open than the wild type enzyme, especially at the N-terminal domain of the protein [Fowler and Zabin, 1983].

5-3. Purification of Fusion Proteins

Quite often difficulties are encountered in purifying proteins that are unstable or difficult to assay. The

construction of fusion proteins with β-galactosidase activity may allow for the co-purification of the target protein with β-galactosidase using a technique for β-galactosidase purification, i.e. a simple one step purification protocol involving the passage of crude extract preparations through a β-galactosidase activity affinity column. Native β-galactosidase bound tightly to the affinity matrix (p-aminobenzyl 1-thio-β-D-galactopyranoside) allowing for over 95% of the enzyme loaded on to the column to be recovered in a purified form. Indeed, binding to the affinity matrix was tight enough that competitive binding site eluters, such as D-lactose, proved ineffective. Only buffers with a pH greater than 10, such as 100 mM sodium borate, were able to successfully elute the adhered protein [Steers et al., 1971].

This technique was used to purify a DNA replication initiator protein (replication initiator protein of plasmid R6K), which has no adequate assay and is unstable, in a β-galactosidase fusion protein form. The fusion protein stabilized the initiator protein without destroying its biological activity [Germino et al., 1983]. Similar results were obtained in the purification of L-SD in the 2PF protein form [Su, 1991].

The same initiator protein was further purified as a fusion construct with chicken proα-2-collagen sequence between the initiator protein and β-galactosidase. Affinity-purified protein was treated with collagenase which digested the

collagen sequence, resulting in separate initiator and β-galactosidase protein molecules [Germino and Bastia, 1984]. The same purification strategy was used in purifying L-SD in the 3PF form [Su, 1991].

The purification of target proteins in a fusion form has been demonstrated to simplify the purification of many proteins that have otherwise been difficult to handle [Ullmann, 1984]. The purified proteins could then be used in enzymological studies and in the raising of antibodies.

II MATERIALS AND METHODS

PART 1. STRAINS AND PLASMIDS

The strains and plasmids used in this study are listed in Table 1.

PART 2. CULTURES AND MEDIA

2-1. Minimal Medium

The minimal medium used has been previously described [Newman et al., 1985a]. Because all the strains used in this study carry a deletion in ilvA and therefore require L-isoleucine for growth, L-isoleucine and L-valine were added to all media at 50 µg/ml each. Unless noted otherwise D-glucose was provided at 2 mg/ml as a carbon source.

2-2. SGL Medium

Medium with a combination of L-serine, glycine and L-leucine as the only carbon source in addition to L-isoleucine and L-valine is called SGL medium. L-Serine, glycine and L-leucine were provided at 2, 0.3 and 0.3 mg/ml respectively unless otherwise noted.

2-3. Other Additions to the Medium

Antibiotics were used at the following concentrations in μg/ml: ampicillin (Amp) 100, kanamycin (Kan) 80, chloramphenicol (Cm) 40.

Table 1. Bacterial Strains and Plasmids

Strain or plasmid	Genotype and relevant characteristics	Source or reference
E. coli K-12		
CU1008	*E. coli* K-12 *ΔilvA*	L. S. Williams
MEW1	*lacZ* derivative of CU1008	Newman *et al.*, 1985b
MEW28	MEW1 *sdaA*::Cm^r	Su and Newman, 1991
MEW84	MEW1::λplacMu9, SGL-, Kan^r	Feng, 1990

MEW128	CU1008 SGL-, isolated by penicillin selection	Newman et al., 1985b
MEW191	CU1008 with MudX causing SGL- phenotype	Newman et al., 1985b
MEW191C	MEW191 SGL-, cured of MudX	Dumont, 1985

Plasmids

pMES22	pBR322 with a 2.6 kb XhoI-BamHI insert carrying sdaA	Su, 1991
pMES27	An in-frame fusion of the sdaA gene at the EcoRI site to the SmaI site of pMC1871	Su, 1991
pMES28	pSD100 carrying the sdaA gene in-frame fusion to the proc α collagen sequence	Su, 1991

pBR322		Bolivar *et al.*, 1977
pBB1	*dnaK,J* carried on pBR322	Dr. Walker
pOF39	*groES,EL* carried on pBR322	Dr. Walker
pDK84	*groES,EL* under control of the *lac* promoter	Dr. Sherman
pMC1871	*lacZ* carried on pBR322	Dr. Storms
pSD100	proc α collagen sequence fused to *lacZ*, λP_R cloned between *EcoRI* and *BamHI*	Moskaluk and Bastia, 1988

PART 3. PROTEIN PURIFICATION

3-1. Purification of the 2PF Protein

Cultures of strain MEW28 pMES27 (p2PF) grown in glucose minimal medium at 37°C with glycine and L-leucine were harvested in late log-phase and disrupted by sonication in TMN buffer (20mM TrisHCl, 10mM $MgCl_2$, 10mM β-mercaptoethanol and 1.6 M NaCl pH7.4). The crude extract was clarified by centrifugation at 12,000 rpm, and the supernatant was loaded on a 1 ml bed volume p-aminobenzyl 1-thio-β-D-galactopyranoside-agarose (ATGA) column, purchased from Sigma Biochemical Co. (product #A1040). The column was then washed with 10 times the bed volume of TMN buffer and eluted with 20% D-lactose in distilled water. To remove most of the lactose, and transfer to an appropriate buffer, the eluate was concentrated approximately 10-fold by centrifugation in a Millipore Centricon 30 microconcentrator, and diluted to the original volume with glycylglycine 50mM pH 8. The concentration step was repeated, diluting a second time in the same volume of glycylglycine.

3-2. Purification of the 3PF Protein

Cultures of strain MEW28 pMES28 (p3PF) grown in glucose-minimal medium at 28°C to early log-phase were transferred to 42°C for 20 min, followed by 2-3 h at 37°C. Enzyme preparations were then made and purified as for the 2PF, with the modification that the enzyme was eluted with 100 mM sodium borate pH 10.5 rather than 20% D-lactose (Ullman et al., 1984). The eluate was concentrated and transferred to 50 mM HEPES buffer pH 8 in the same way as was used to transfer the 2PF to glycylglycine.

3-3. Chromatography by FPLC

Separations were carried out with the use of Pharmacia P-500 pumps operated by a Pharmacia gradient programmer GP-250 Plus, using a Superose 12 column, made by the same manufacturers.

PART 4. ENZYME ASSAYS

4-1. Standard L-SD Assays

L-SD was assayed as described previously in toluene-treated whole cells (Isenberg and Newman,1974; Pardee and Prestidge 1955). One unit of L-SD, as measured in the whole

cell assay (in vivo) is defined as the amount of enzyme which catalyzes the formation of 1 µmol of pyruvate in 35 min at 37°C.

Inactive L-SD was assayed in vitro in either HEPES or glycylglycine buffer (both 50 mM, pH 8) as previously described [Newman et al., 1985a] in a total assay volume of 170 µl containing 55 mM L-serine, 1.8 mM $FeCl_3$, and 4.45 mM DTT, unless otherwise noted. This mixture was allowed to incubate at 37°C for 35 min after which the reaction was stopped with dinitrophenylhydrazine in HCl (DNPH) and the keto acid was determined as previously described [Newman and Kapoor, 1980]. One unit of L-SD is defined as the amount of enzyme which produced 1 µmol pyruvate per 35 min at 37°C.

4-2. L-SD Activation In Vitro

Inactive L-SD prepared in 50 mM HEPES (pH 8) was activated in a total volume of 170 µl containing 5 mM L-serine, 1.8 mM $FeCl_3$, and 4.45 mM DTT, at 37°C for 15 minutes.

4-3. L-SD LDH/NADH Coupled Assay

The protocol for the coupled assay is similar to that used by other investigators [Farias et al., 1991] with the following modifications. L-SD was first activated (section 4-2). An aliquot of the incubated mixture (10 µl) was then

pipetted into a cuvette at 37°C in a total volume of 700 µl containing 55 mM L-serine, the optical density was monitored at 340 nm for 5 minutes, allowing the absorbance reading to stabilize. NADH (0.3 mM), LDH (0.33 µg/ml), and L-serine (maintaining the L-serine concentration at 55 mM) were then added to give a final assay volume of 750 µl and the rate of the activated L-SD was then followed by following the decrease in absorbance at 340 nm.

4-4. β-Galactosidase Assay

β-Galactosidase activity was assayed by the method of Miller and is expressed in his units [Miller, 1972].

4-5. Protein Assay

Protein concentration was determined by the method of the Bradford assay [Bradford, 1976].

4-6. ELISA Assay

Immulon microtiter plates purchased from Canlab were adsorbed with antigen and probed with antibodies for antibody reactive material according to the instructions in Antibodies A Laboratory Manual [Harlow and Lane, 1988].

The microtiter plates were probed for primary antibody

(2PF immune serum) reacting material with horseradish peroxidase-lablled Goat anti-Rabbit antibodies purchased from Sigma using 4-Chloro-1-naphthol, also purchased from Sigma, according to the instructions in Antibodies A Laboratory Manual [Harlow and Lane, 1988]. The Microtiter plate was then read with a MR 300 Microplate Reader purchased from Canlab.

PART 5. PROTEOLYTIC DIGESTIONS

5-1. Collagenase Digestion

Purified collagenase (20 units/mg) from Achromobacter iophagus was purchased from Boehringer Mannheim Co. and dissolved in 0.01M Tris-HCl pH 7.4 containing NaCl (0.25M), CaCl2 (0.01M) and 2-mercaptoethanol (0.01M) at a concentration of 1 mg/ml (Germino and Bastia, 1984). To digest the 3PF, 50 µg of 3PF protein in 50 µl 50 mM HEPES pH 8 were incubated with 0.1 ul of the collagenase stock solution at 30 ^{0}C for 15 min.

5-2. Trypsin Digestion

Trypsin, purchased from Boehringer Mannheim Co., was incubated at 37^{0}C for the time intervals and at the protein concentrations given in the legend (Fig. 13), with L-SD that had been activated as in 4-2 or suspended in the activation

mix and kept on ice for the duration of the activation.

PART 6. ELECTROPHORETIC TECHNIQUES

6-1. Gel Electrophoresis and Staining

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Bio-Rad Mini Protein II dual slab cell according to the instructions of the manufacturer. Silver staining was done with the Biorad Silver Staining Kit, also according to their instructions. Coomasie staining was performed as described in the cloning manual [Maniatis et al., 1982].

6-2. Western Blot Analysis

SDS-PAGE was performed as described in 6-1. The gels were then transferred to a Bio-Rad Mini Protein II dual slab cell electrobloter and were electrophoretically transferred to .45 μm pore size nitrocellulose membranes (purchased from Millipore Co.) according to the instructions of the Millipore Company.

The membranes were probed for primary antibody (2PF immune serum)-reacting material with horseradish peroxidase labelled Goat anti-Rabbit antibodies purchased from Sigma using 4-Chloro-1-naphthol purchased from Sigma, according to

the instructions of Antibodies A Laboratory Manual [Harlow and Lane, 1988].

6-3. Edman Degradation

The Edman degradation was performed according to the method of Matsudaira (1987) with modifications noted as follows. The 2PF protein, purified by affinity chromatography, was subjected to SDS-PAGE, and then transferred from the gel to Polyvinylidene difluoride (PVDF) membranes purchased from Millipore Co. using a Biorad Minigel Electroblotter according to the manufacturers instructions. The PVDF membranes were washed in deionized water for 5 min., stained with 0.1% Coomassie Blue R-250 in 45% methanol for 2 min. and destained in 45% methanol with 10% acetic acid for 5 minutes. The bands corresponding to the 2PF molecular weight were cut out and sent for amino acid sequencing to Dr. Max Blum at the University of Toronto, who carried out the sequencing on an Applied Biosystems 470A gas phase sequencer with an in-line 120A PTH-amino acid analyzer.

PART 7. THE PRODUCTION OF ANTIBODIES

The immunization and bleeding of animals was performed by technicians at the McGill Animal Resources Centre, 3655

Drummond Street, Room 1416, Montreal, Que., H3G 1Y6.

2PF protein was purified as described in 3-1 and mixed by vigorously vortexing with an equal volume of complete Freunds adjuvant purchased from Calbiochem, to give a final antigen concentration of about 3 mg/ml with which 6 rabbits were injected subcutaneously, 6 injections at approximately 300 µl of antigen/complete adjuvant mixture per injection. Incomplete Freunds adjuvant purchased from Calbiochem and mixed with antigen in the same way as the complete adjuvant and injected about 4 weeks after the initial injections.

About 2 weeks later 100 ml of blood was obtained from a cardial puncture of each rabbit. The animals were sacrificed by this procedure. After collection the blood was allowed to clot for 60 min at 37°C and placed at 4°C overnight. The serum was removed from the clot by centrifugation at 10,000g for 10 min at 4°C and stored at -20°C.

PART 8: GENETIC TECHNIQUES

Plasmid isolations and DNA transformations were performed as described in the cloning manual (Maniatis et al., 1982).

III RESULTS

The work in this thesis is devoted towards an understanding of the enzyme L-serine deaminase (L-SD), its purification, activity and activation *in vivo* and *in vitro*. My work is based on the results of H. Su who cloned and sequenced the *sdaA* gene, which he showed encodes the protein L-SD [Su, 1991]. My work provides additional evidence that *sdaA* is the structural gene for L-SD and characterizes the enzyme and its activation.

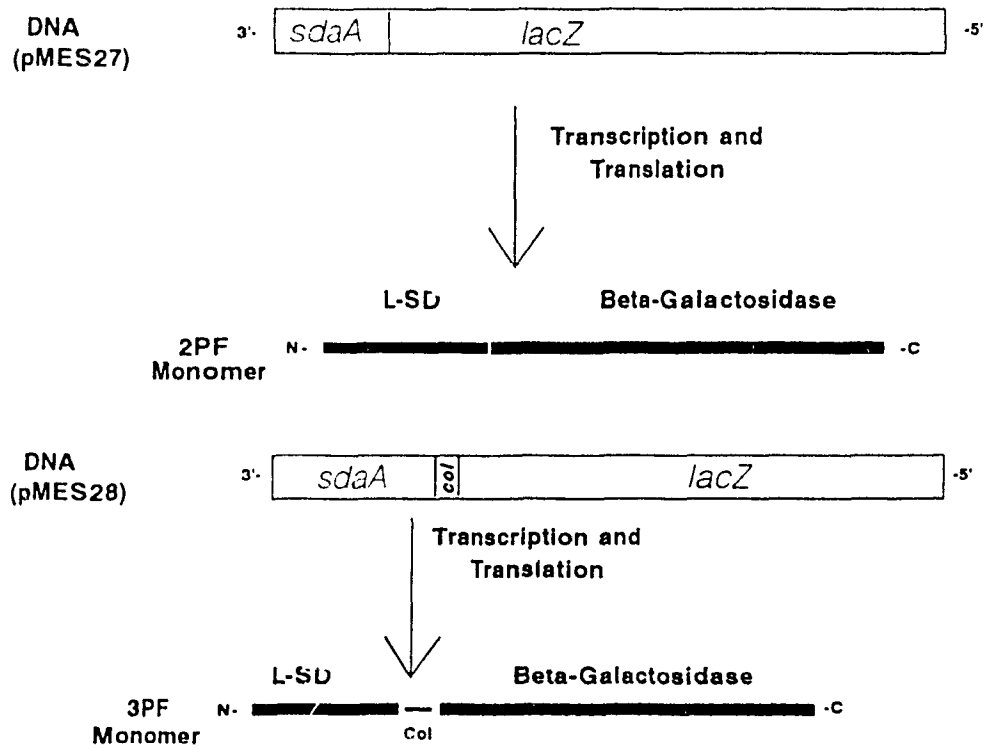
PART 1. THE PURIFICATION OF L-SD

As the first step in my investigation I wanted to obtain pure L-SD for experimental analysis. To accomplish this, I used a purification strategy worked out by H. Su [Su, 1991] in which he constructed a gene with *sdaA* and *lacZ* sequences fused in frame without a stop codon between them. H. Su constructed two plasmids with the fused genes, pMES27 and pMES28, coding for the 2PF and 3PF proteins respectively (defined below). These are large proteins carrying both L-SD and β-galactosidase activities. This construct allows procedures for the purification of β-galactosidase to be used for the co-purification of L-SD [Su, 1991]. Since the gene fusions were constructed on a high copy number vector, pBR322, strains carrying these plasmids overproduce the fusion proteins,

resulting in a production of a lot of protein in a form ideal for purification.

The 2PF protein is composed of an N-terminal L-SD molecule fused to a C-terminal β-galactosidase molecule. The 3PF protein is composed of an N-terminal L-SD molecule fused to a collagen sequence which is fused to a C-terminal β-galactosidase molecule (see Fig. i). The collagen sequence is 63 amino acids in size and contains 6 consensus cleavage sites for collagenase (-Pro-X-Gly-Pro-Y-) [Germino and Bastia, 1984].

FIG. i



The 2PF and 3PF proteins can, in principle, be purified on any β-galactosidase affinity column, after which the 3PF can be treated with collagenase, resulting in the cleavage of the protein to give free L-SD and β-galactosidase protein molecules [Su, 1991]. H. Su and I both used an ATGA (p-aminobenzyl 1-thio-β-D-galactopyranoside agarose) β-galactosidase affinity column which is effective and relatively inexpensive.

1-1. Purification of the 2PF Protein

Plasmids were transformed into strain MEW28 because this strain has a deletion in sdaA and thus makes no L-SD. Therefore all L-SD activity must be derived from the plasmid-carried fusion. The 2PF protein was purified from extracts of the sdaA deletion carrying the 2PF plasmid (hereafter called the 2PF strain). The 2PF strain was grown in minimal medium to avoid the presence of the sdaB gene product, a second L-SD (L-SD#2) which is only made in LB grown strains [Su and Newman, 1991].

Since both enzyme activities are expected to be on a single protein, I expected L-SD and β-galactosidase to co-purify. In crude extracts, L-SD is in an inactive state that can be activated in vitro with Fe and DTT [Newman et al., 1985a]. In all the steps presented in the purification of the 2PF, this requirement for L-SD activation remained.

TABLE 2: Purification of the 2PF Protein on the ATGA Column

Purification Step	% Act. L-SD	Total Prot. (mg)	β-gal Spec. Act. (U/mg)	L-SD Spec. Act.** (U/mg)	β-gal to L-SD Ratio	L-SD Pur. Fold
(1) Crude Extract	100	118	847	4.15	204	1
(2) Extract Through Column	11	80	138	0.7	197	0.17
(3) TMN Washings	-	39	3.8	*NDT	-	-
(4) Elution, Resuspension	85	1.5	56533	277	204	66.7

Cells of strain MEW28 carrying plasmid pMES27 were grown in glucose-minimal medium, harvested, and sonicated in TMN buffer (step 1). 10 ml of this extract was loaded on to a 1 ml bed volume ATGA column, and the material passing through the column was collected (step 2). The column was washed with 15 ml of TMN (step 3), followed by elution with 20% lactose. The protein eluted with the lactose was resuspended and concentrated in 50mM glycylglycine pH 8, using Centricon 30 microconcentrators (step 4).

The results given are from one experiment, and are typical of several.

* NDT stands for none detected

** The standard L-SD assay was used (see Materials and Methods)

The data in Table 2, a 2PF purification table that presents L-SD and ß-galactosidase activities, shows that the two enzymes do indeed co-purify, with the ß-galactosidase to L-SD ratio remaining constant throughout the purification, and both activities being purified about 67 fold. 20% D-lactose was used for elution in this experiment, 100 mM sodium borate was also used for elution on several occasions, with no observable difference.

1-2. Purification of the 3PF Protein

Extracts from the same cells (strain MEW28) carrying the plasmid coding for the 3PF were treated as described in section 1-1 except that in the experiment presented in Table 3, the fusion protein was eluted with 100 mM sodium borate pH 10.5 as suggested in the original method [Ullmann, 1984], rather than with 20% D-lactose. Though both buffers eluted the 2PF protein efficiently, lactose eluted the 3PF protein much less efficiently (Table 4) than the borate. Approximately 2.5 times more protein was eluted with 100 mM sodium borate than with the 20% lactose. H. Su had also experienced difficulty eluting 3PF with lactose [Su, 1991]. That the 3PF protein carries both L-SD and β-galactosidase molecules is shown by the fact that the two activities again co-purified as is evidenced by following the consistency of the ß-galactosidase to L-SD ratios through Tables 3 and 4.

TABLE 3: Purification of the 3PF Protein with Sodium Borate on the ATGA Column

Purification Step	% Act. L-SD	Total Prot. (mg)	β-gal Spec. Act. (u/mg)	L-SD Spec. Act. (u/mg)	β-gal to L-SD Ratio	L-SD Pur. Fold
(1) Crude Extract	100	103.5	3480	17.4	200	1
(2) Extract Through Column	37	76.5	1717	8.8	195	0.51
(3) TMN Washings	6	30	750	3.9	192	0.22
(4) Elution, Resuspension	47	3.7	44758	230	195	13.2

TABLE 4: Purification of the 3PF Protein with 20% D-Lactose on the ATGA Column

Purification Step	% Act. L-SD	Total Prot. (mg)	β-gal Spec. Act. (U/mg)	L-SD Spec. Act. (U/mg)	β-gal to L-SD Ratio	L-SD Pur. Fold
(1) Crude Extract	100	103.5	3480	17.4	200	1
(2) Extract Through Column	35	76	1680	8.3	202	0.48
(3) TMN Washings	6	30	740	3.5	211	0.20
(4) Elution, Resuspension	18	1.3	46154	246	188	14

Cells of strain MEW28 carrying plasmid pMES28 were grown in glucose-minimal medium, harvested, and sonicated in TMN buffer (step 1, Table 3). 9 ml of this extract was loaded on to a 1 ml bed volume ATGA column, and the material passing through the column was collected (step 2, Table 3). The column was washed with 15 ml of TMN (step 3, Table 3), followed by elution with 100mM sodium borate [Ullmann, A., 1984] (Table 3). The protein eluted was resuspended and concentrated in 50mM HEPES pH 8, using Centricon 30 microconcentrators.

The purification presented in Table 4 was performed as in Table 3, with elution by 20% D-lactose instead of 100 mM sodium borate.

The results given in Tables 3 and 4 are from single experiments and are typical of several.

As in the case of the 2PF protein purification, Fe and DTT were required in all steps for L-SD activation. L-SD as part of the 3PF was purified 13 or 14 fold (Tables 3 and 4). These results are considerably lower than the 67 fold purification of the 2PF in Table 2. This lower degree of purification results from the 3PF plasmid expressing its product at a higher level than the 2PF plasmid, due to the inactivation of a temperature sensitive λ repressor [Su, 1991] when the 3PF plasmid-carrying cells are transfered to a high temperature.

The L-SD specific activity for the 3PF (230 and 246 u/mg, Tables 3 and 4 respectively) appears to be lower than the specific activity for the 2PF protein (277 u/mg, Table 2). However, the 3PF contains an additional 66 amino acids when compared with the 2PF, and thus would weigh about 7 kDa, or about 5% more than the 2PF. If this molecular weight difference were taken into account when comparing the 2PF and 3PF specific activities, it would raise the 3PF specific activity somewhat (about 10 U/mg if there were no contaminating proteins in the purified preparations). Also, the purification is not perfect in that some contaminants co-purify along with the fusion proteins, a fact that becomes evident on examination of SDS-gels of the fusion protein (eg. Fig. 1), and although the conditions during each purification are nearly identical, each separate purification will probably yield differing levels of protein contaminants, thus the specific activity will vary somewnat from purification to

purification.

When comparing the % recovery of the 2PF and 3PF purifications, in the case of the 2PF protein, 85% of the overall enzyme activity was recovered but with the 3PF only about 47% was recovered (eluted under optimal conditions, Table 3). This is due to a significant percentage of 3PF protein passing through the affinity column without adhering (steps 2 and 3 of Tables 3 and 4). With the 2PF protein, much less fusion protein passed through the column in these steps (Table 2).

The fraction of 3PF protein that passed through the column was not due to overloading. I never loaded more than 400 000 ß-galactosidase activity units on to the ATGA column, and the amount of ATGA matrix that I used for the purifications can bind over 4 million ß-galactosidase activity units [Sigma technical communication]. Additionally, experiments were performed by loading about 5, 10 and 15 times less protein with the same % of activity passing through the ATGA column.

1-3. The Separation of L-SD and β-Galactosidase From Pure 3PF Protein

If the 3PF protein has the expected structure (Fig. i) then incubation with collagenase should cut the protein into 2 polypeptides, of which one would be L-SD and the other would be ß-galactosidase. To test this, affinity-purified 3PF

protein was incubated with collagenase and then subjected to both SDS-PAGE and FPLC gel filtration analysis.

On SDS-PAGE, affinity purified 3PF migrated with a molecular weight greater than 116 kDa (Fig. 1, lane B). After treatment with collagenase, the 3PF band disappeared and was replaced by two lower molecular weight bands at 116 and 48.5 kDa (lane C). These correspond to the molecular weights of the β-galactosidase monomer [Ullmann, 1984] and L-SD [Su et al., 1989] respectively.

The effect of collagenase treatment was also investigated using FPLC gel filtration analysis, which separates proteins by size under non-denaturing conditions. Since the proteins are not denatured, eluted fractions can be assayed for enzyme activity. ATGA-purified 3PF was chromatographed on a Superose 12 FPLC column (Fig. 2). The purified 3PF peak eluted at fraction #14, which contained both L-SD and β-galactosidase activities. After treatment of the affinity-purified 3PF with collagenase, the activities shifted to lower molecular weight fractions (Fig. 2). The β-galactosidase activity now eluted at fraction #20, and the L-SD peak at fraction #23. Fe and DTT were required in all steps for L-SD activation.

Thus treating the 3PF protein with collagenase resulted in the formation of two lower molecular weight proteins as shown by both SDS-PAGE (Fig. 1) and FPLC gel filtration (Fig. 2) experiments. The higher molecular weight fraction showed β-galactosidase activity and the lower, L-SD activity.

Figure 1: SDS-PAGE of Affinity Purified 3PF with and without Collagenase Treatment

7.5 % SDS gels were run following the manual of BIO-RAD, and then stained with the Silver Staining Kit purchased from BIO-RAD. Lanes A and D contain molecular weight markers. Lanes B and C were each loaded with 5 µg of affinity-purified 3PF protein and affinity-purified collagenase treated (as described in Materials and Methods) 3PF protein respectively.

This figure represents one experiment and is typical of many.

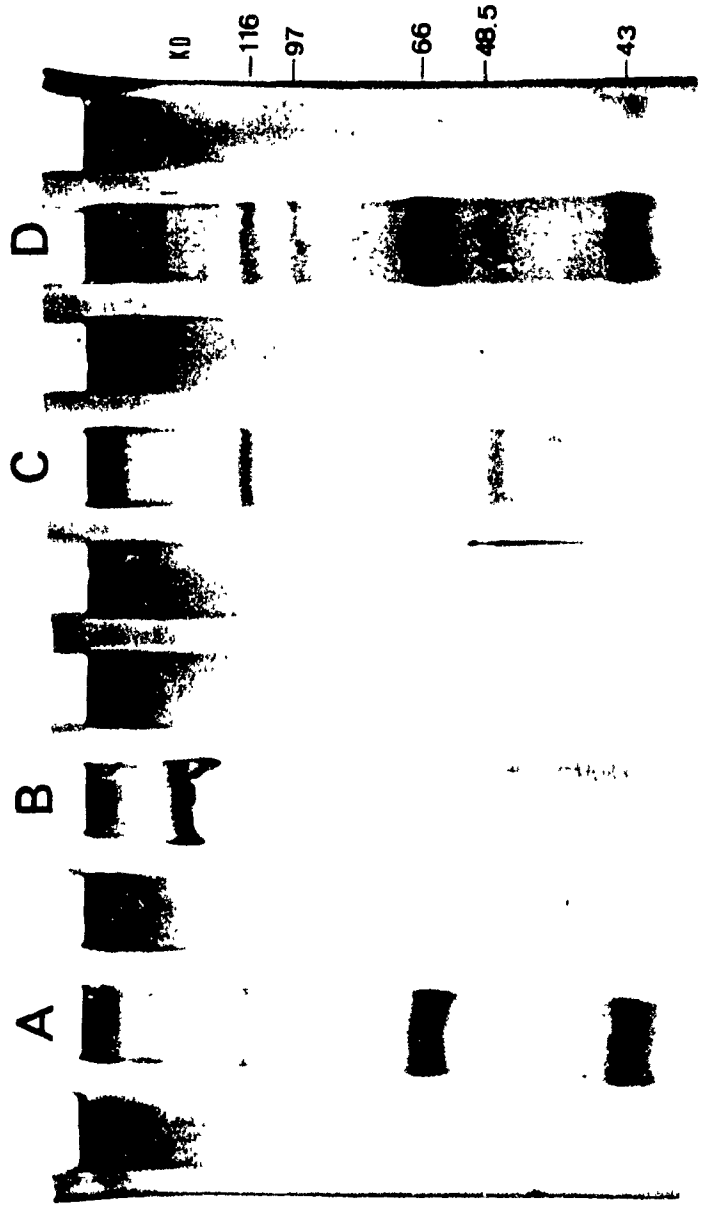
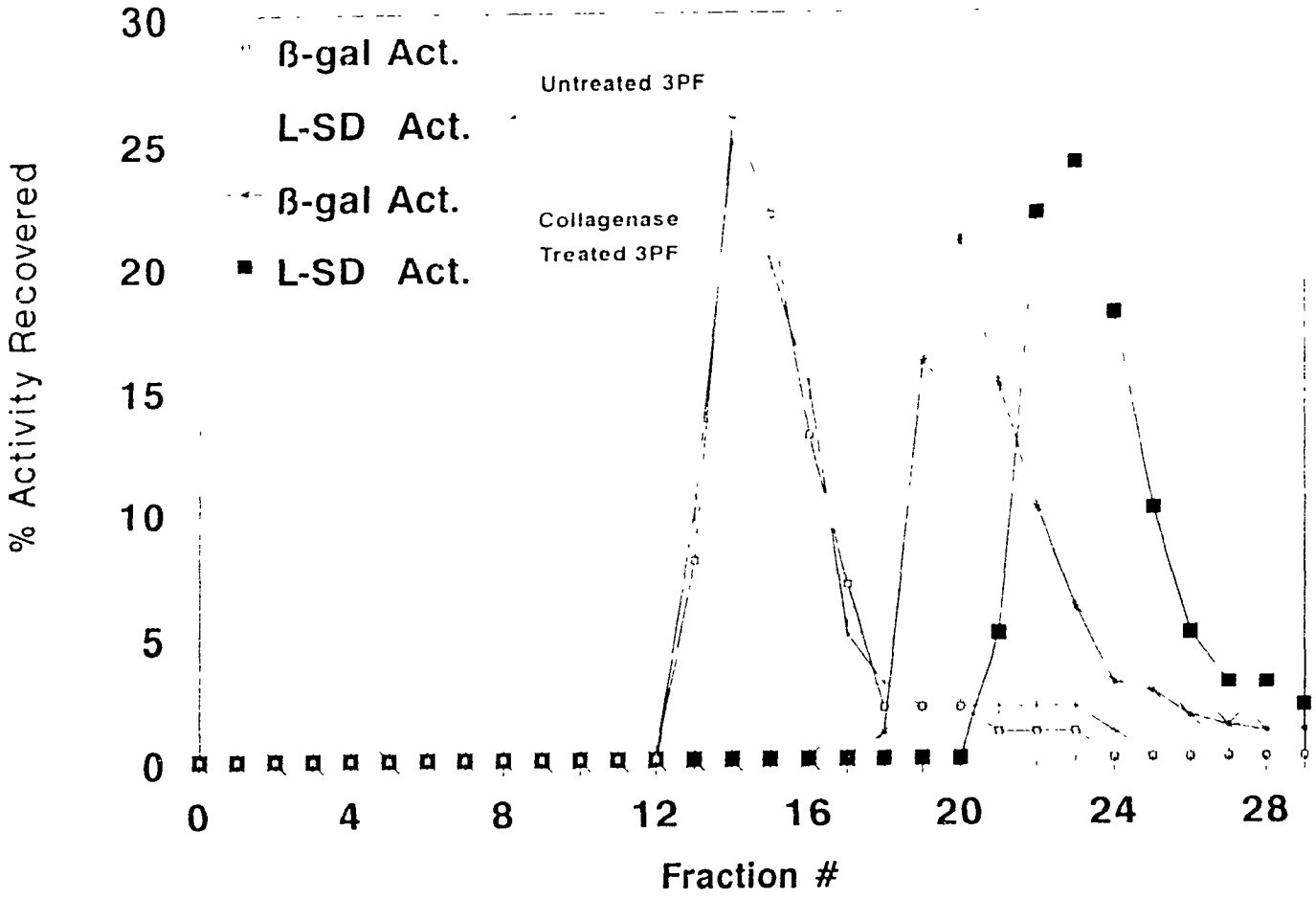

KD
116
97
66
48.5
43
D
C
B
A

Figure 2: FPLC Analysis of Affinity-Purified 3PF Protein before and after Collagenase Treatment

Equal quantities of affinity-purified 3PF protein and affinity-purified collagenase treated 3PF protein were loaded on a FPLC Superose 12 FPLC column equilibrated with a 50 mM HEPES pH 8 buffer. Using a flow rate of 0.5 ml/min, fractions were collected at 1 minute intervals. The fractions were assayed for L-SD (standard L-SD assay as described in Materials and Methods) and β-galactosidase activities as described in Materials and Methods. The data is presented in terms of the % activity recovered in each fraction compared to the total activity loaded on to the FPLC.

These results are from one experiment and are typical of several.

Molecular weight markers Aldolase and Egg Albumin were chromatographed in separate runs under the same conditions. They consistently elute in the volumes indicated in Figure 2.



MOLECULAR WEIGHT MARKERS:
Aldolase 158 KD (fraction 22)
Egg Albumin 45 KD (fraction 24)

From an analysis of L-SD activity throughout the purification procedure an initial observation on L-SD can be made. In experiments until now there has been the possibility that for in vitro L-SD activity another protein or co-factor may be required which could be present in bacterial extracts. Throughout the purification procedure, Fe and DTT are always required for L-SD activity. However, there are no additional requirements for anything other than Fe and DTT even after FPLC chromatography. Therefore, if a co-factor plays a role in L-SD activity and/or activation, then it must co-purify with L-SD.

PART 2. VERIFICATION OF THE L-SD N-TERMINAL AMINO ACID SEQUENCE

In order to verify that the sdaA sequence corresponds to that of the 2PF protein, i.e. that I am indeed working with the protein coded for by sdaA, an Edman Degradation was performed on the 2PF protein. An Edman Degradation determines the amino acid sequence of a polypeptide, starting at its N-terminal. By comparing the actual amino acid sequence with the amino acid sequence deduced from the DNA sequence, a conclusion can be made as to whether I am working with the sdaA gene product, while at the same time providing additional supporting evidence that the DNA sequence is correct. Since the protein fusion is constructed with the L-SD protein molecule at the N-terminal, the fusion protein can be used in this procedure without further processing.

2-1. Edman Degradation on 2PF Protein

For the Edman Degradation, 2PF protein was purified from extracts as in Table 2. The affinity column does not produce pure 2PF. A few contaminants apparently always co-purify with the 2PF. These low level contaminants would interfere in the Edman Degradation.

In order to obtain a purer sample of protein for sequencing, the affinity-purified 2PF protein was run on SDS-PAGE. The proteins were then electroblotted on to PVDF membranes. The membranes were lightly stained with

Figure 3: The Edman Degradation of 2PF Protein

The Edman Degradation was performed by Dr. Blum at the University of Toronto, Biochemistry Department. (1) is the DNA sequence of L-SD as determined by Dr. Su [Su et al., 1989]. (2) is the expected amino acid sequence deduced from the DNA sequence at the ATG start site [Su et al., 1989]. (3) is the expected amino acid sequence deduced from the DNA sequence starting from a GTG start site. (4) are the results obtained from the Edman Degradation.

(1) DNA

Sequence : GTG ATT AGT CTA TTC GAC ATG TTT AAG GTG GGG

(2) ATG Start : N- Met Phe Lys Val Gly

(3) GTG Start : N- Met Ile Ser Leu Phe Asp Met Phe Lys Val Gly

(4) Edman : N- ? Ile ? Leu Phe Asp Met Phe

Coomasie Blue, and the band which corresponded to the 2PF was cut out and sent for sequencing [Matsudaira, 1987].

The results of the Edman degradation (Fig. 3) show that the translational start site that was proposed [Su et al., 1989] was incorrect. The amino acid sequence obtained from the Edman degradation does correspond to the amino acid sequence which is derived from the DNA sequence. However, the real transcriptional start site is 18 bp, 6 amino acids, upstream of the start site originally proposed.

The Edman data provided additional data confirming that I was indeed working with the protein coded by sdaA, and I could proceed with my investigation.

PART 3. RAISING ANTIBODIES AGAINST L-SD

Raising antibodies against L-SD would be important in my investigation. With an antibody to L-SD, I could identify L-SD in bacterial extracts and in purified protein preparations. The antibodies would also allow me to study the size of L-SD *in vivo*, and address the question of whether or not the molecular weight of L-SD changes when it is activated *in vivo* or *in vitro* (Part 7 of Results).

3-1. 2PF Protein Used as the Antigen to Produce Antibodies to L-SD

In order to obtain antibodies which were specific for L-SD, rabbits were injected with 2PF purified as in Table 2, and chromatographed on a Superose 12 FPLC column as was performed with the 3PF in Fig. 2. The 2PF protein preparations always contain some low level contaminants (this is why a great deal of extra effort was put in to preparing as pure a sample as possible in Part 2 for the Edman degradation). In hindsight, at this point I should have overloaded SDS-gels with the antigen in order to identify how much, and at what molecular weight contaminants were present.

Using the 2PF as the antigen, rabbits were initially injected with an antigen/Freund's Complete Adjuvant mixture. They were given booster shots with an antigen/Freund's Incomplete Adjuvant mixture, and bled for serum as described

in Materials and Methods. Five different rabbits were used (00, 01, 03, 84 and 85) with approximately 50 ml of serum obtained from each rabbit.

3-2. Testing for Antibodies Against 2PF Protein

After bleeding the rabbits for serum, it was necessary to test for the presence of antibodies against the antigen, 2PF. I decided that the best way to do this was to perform an ELISA assay using 2PF protein as the antigen.

The ELISA assay that I performed (see Materials and Methods) tested for the presence of antibodies bound to antigen by using a secondary enzyme (horseradish peroxidase)-linked antibody. This secondary enzyme-linked antibody (goat anti rabbit) recognizes the initial antibody raised in rabbits. The presence of the complex can then be determined by adding the enzyme's substrate, chloronaphtol. The colored product can be quantified spectrophotometrically at 492 nm.

The results of the ELISA (Fig. 4) indicate that there are antibodies present against proteins in a purified 2PF sample up to a titer of 1/5 000 (these results are for serum sample 00). Although I would expect a portion of the antibodies to be specific for the 2PF protein, the antibodies may also recognise a purification contaminant. If I had a purification contaminant in the 2PF preparation then I would have initially

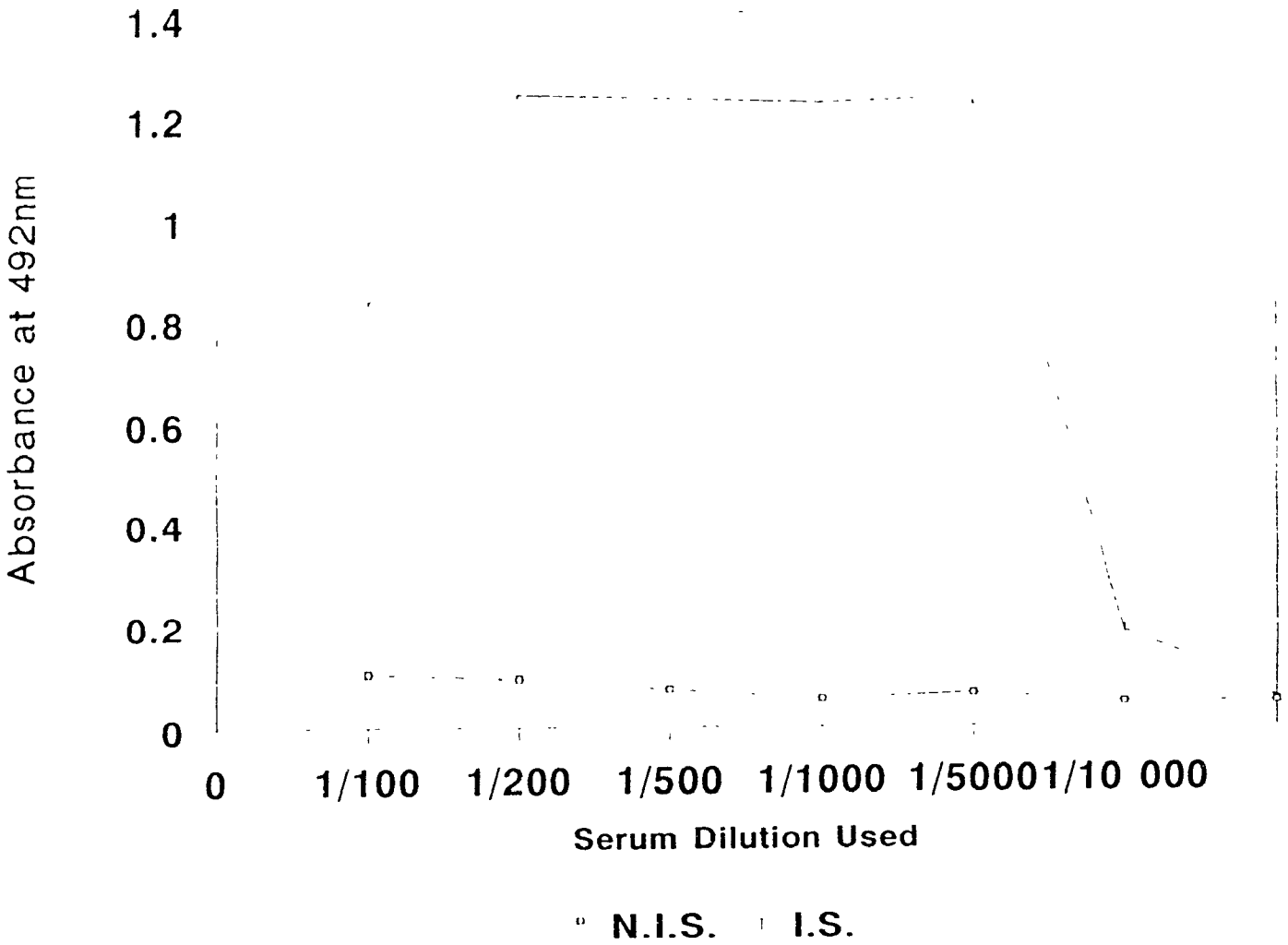
Figure 4: ELISA Assay with 2PF Protein as the Antigen

ELISA assays were performed as described in Materials and Methods at the given serum dilutions, with .05 µg of purified 2PF protein (from Table 1) used as antigen.

Immune serum (IS) 00 was used in this experiment as was non-immune serum (NIS) obtained from the same animal prior to injection with antigen.

The experiment also included ELISAs with no antigen, and antigen with secondary antibody but no IS. Neither gave a coloured product (data not shown).

The results given are from one experiment and are typical of three.


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 492nm
0
1/100
1/200
1/500
1/1000
1/5000
1/10 000
Serum Dilution Used
N.I.S.
I.S.

injected the rabbits with 2PF protein as well as the contaminant. Therefore, in the ELISA, which was performed with the same 2PF protein, I may be detecting a contaminant as well as the 2PF protein. Similar results were obtained for the other four serum samples 01, 03, 84 and 85.

3-3. Testing for Antibodies Against L-SD

Knowing that I had antibodies to the 2PF preparation, I then proceeded to determine if I had antibodies against L-SD itself. Antibodies against L-SD might interfere with enzyme activity. To test this I carried out L-SD assays with and without antibodies present. Table 5 presents the results of a typical assay. In the presence of serum, L-SD activity was lowered to 28% of its original level. However, there could be many factors in the serum that may have inhibited L-SD activity, including β-galactosidase antibodies.

For additional evidence to show that I had antibodies specific for L-SD, I looked for antibody-reacting material in E. coli extracts which were or were not expected to contain the L-SD protein. Strain MEW28 (sdaA::Cm^r) has a Tn 9::Cm^r insertion in the sdaA gene and a deletion in the lac operon and so would not be expected to produce L-SD or β-galactosidase [Su, 1991]. I obtained the MEW28/pMES22 strain by transforming MEW28 with plasmid pMES22 which carries the L-SD gene [Su, 1991]. Since plasmid pMES22 carries the sdaA

TABLE 5: The Inhibition of Pure 2PF L-SD Activity by Serum Sample 00

	No Serum (A)	With Serum 00 (B)	Non Immune Serum (C)
L-SD Activity u/µg 2PF	.250	.070	.248

The above assay was done by first activating the L-SD of the purified 2PF protein, as described in the Materials and Methods, except that a 50 mM pH 8 glycylglycine buffer was used instead of a 50 mM HEPES pH 8 buffer. An aliquot of the activated mixture was then placed into a 50 mM glycylglycine buffer, pH 8, containing 55 mM L-serine without any serum (A), serum sample 00 at a 1/5000 dilution (B), or non immune-serum (C) at a 1/5000 dilution, all in a final volume of 170 µl. This mixture was incubated at 37°C for 35 min and pyruvate formation was assayed using DNPH as in the standard L-SD assay (see Materials and Methods).

The assay was performed in this way in order to dilute out the iron and DTT which may degrade the antibodies.

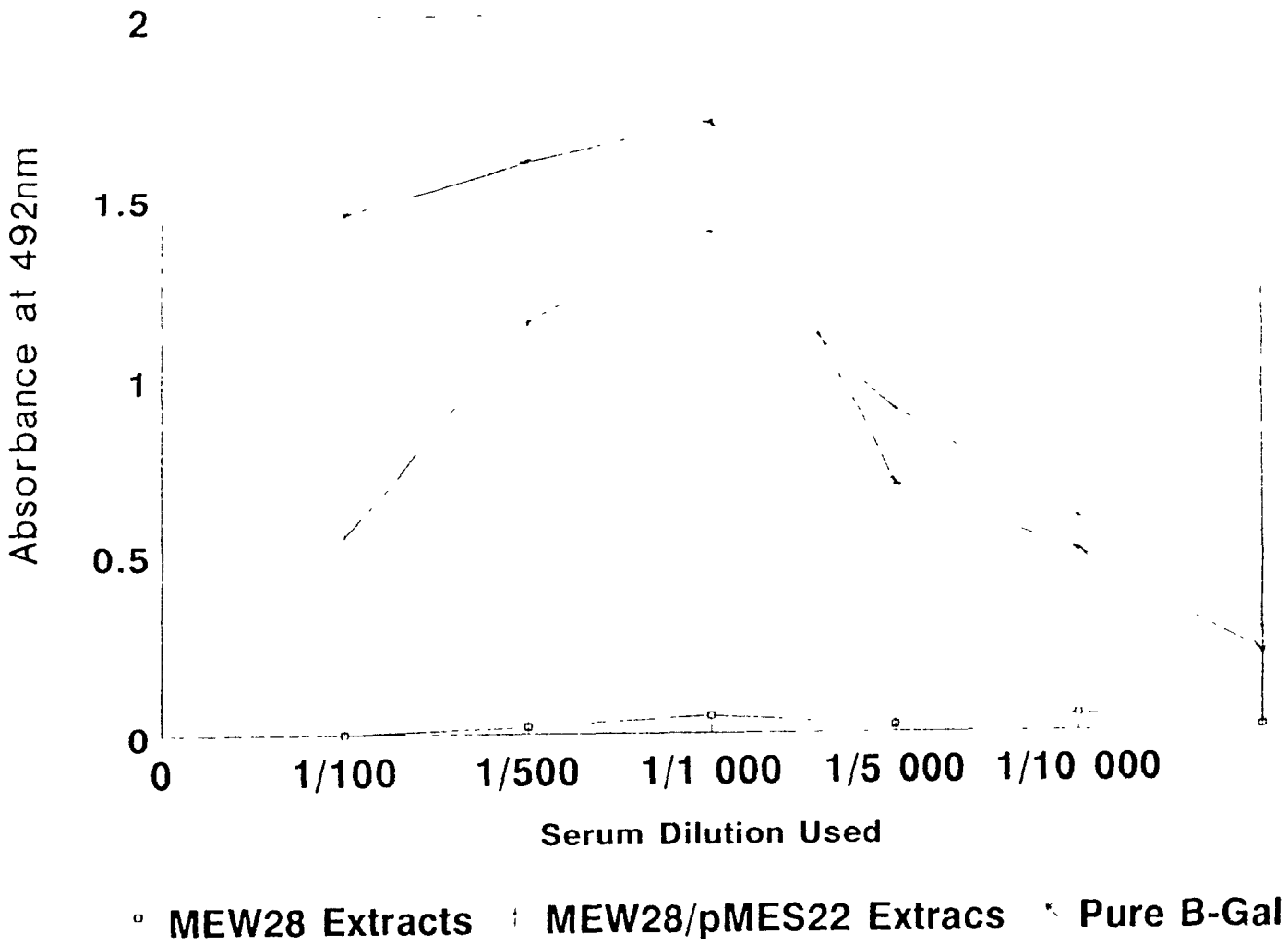
The results are from one experiment and are typical of several.

Figure 5: ELISA Assay with and without L-SD in Extracts as Antigen

ELISA assays were performed as described in Materials and Methods, with .05 µg of extract obtained from either MEW28, MEW28/pMES22, or pure ß-galactosidase protein purchased from Sigma used in the ELISA plate wells.

Immune serum 00 was used in this experiment. Values are corrected for reactions with non-immune serum. The experiment also included ELISAs with no antigen and antigen with secondary antibody but no immune serum. Neither gave a coloured product (data not shown).

The results given are from one experiment and are typical of three.


2
1.5
1
0.5
0
Absorbance at 492nm
0
1/100
1/500
1/1 000
1/5 000
1/10 000
Serum Dilution Used
MEW28 Extracts
MEW28/pMES22 Extracs
Pure B-Gal

gene it should direct synthesis of the L-SD protein in large amounts and no β-galactosidase protein would be present in the extracts to complicate the interpretation. I also used pure β-galactosidase in the ELISA assay knowing that there should also be antibodies against the β-galactosidase protein. The results of this experiment (Figure 5) showed that both the MEW28/pMES22 extract and pure β-galactosidase reacted with antibody but MEW28 extract did not. The MEW28 extract having no β-galactosidase or L-SD proteins was not expected to react with the antibodies. The MEW28/pMES22 extract having antibody reacting material suggests that a protein encoded for by the plasmid pMES22 is responsible for the antibody reaction and only two proteins are encoded for by this plasmid, L-SD and β-lactamase the protein responsible for the plasmid ampicillin resistance selection [Su, 1991].

SDS-PAGE experiments using ATGA-purified collagenase-treated 3PF protein, MEW28 extracts and MEW28/pMES22 extracts were conducted and the proteins blotted onto nitrocellulose and probed for L-SD with the antibodies. The results are shown in Fig. 6. The MEW28 extract (lane F) showed no reaction with the antibodies. The MEW28/pMES22 extract (lane E) showed a band which corresponds to the L-SD band in lane B, between the 43 and 66 kDa molecular weight markers. The only other protein encoded for by the plasmid pMES22 other than L-SD is β-lactamase, which has a molecular weight of 25 kDa [Richmond, 1975] and thus can not be responsible for the band observed between 43 and 66 kDa.

In subsequent gels (Fig. 11, gel 1) a band is present at about 50 kDa before treatment of the 3PF with collagenase.

Although this appears to be 3PF that has somehow disassociated to separate L-SD and β-galactosidase molecules without collagenase treatment (the β-galactosidase monomer band is also present at 116 kDa) this may be a protein purification contaminant. I therefore may have injected rabbits with this contaminant, if it were present in the 2PF preparations, and obtained antibodies to it. Thus the antibody band observed between 43 and 66 kDa of Fig. 6 may simply be this contaminant. However, if this were the case the contaminant should also have been present in the MEW28 extract, MEW28 being the host strain for both the 2PF and 3PF plasmids so any contaminant would have originated from its extracts, but no antibody signal was observed against this extract in both the blot (Fig. 6, lane F) and the ELISA (Fig. 5) experiments. However, it could be that the contaminant is below detectable limits in these examples and co-purifies with the fusion proteins by associating with them thus becoming detectable only in purified preparations.

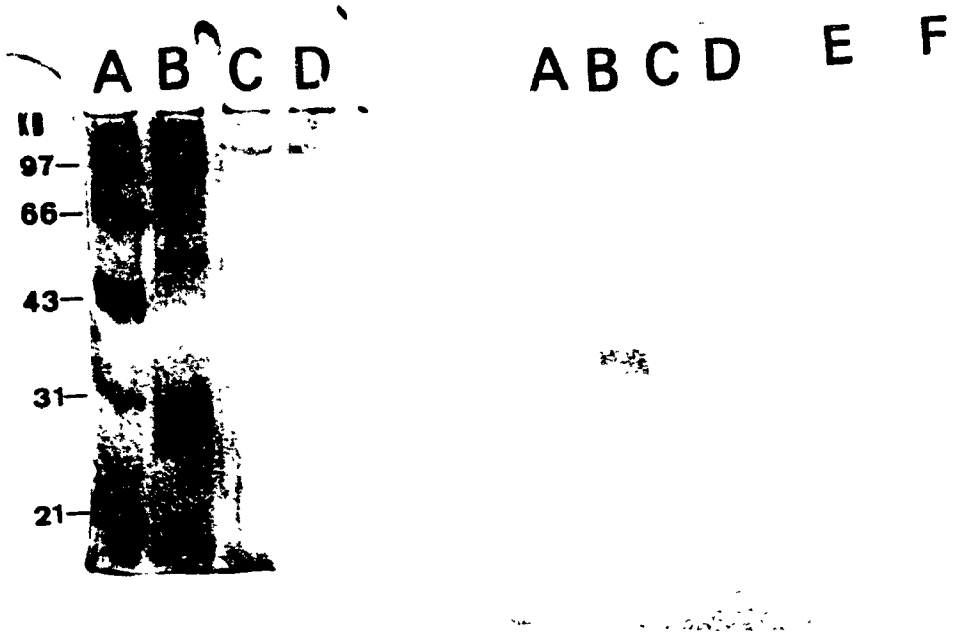
These experiment indicate that I have antibodies to the 2PF preparation and may have antibodies to L-SD, which appears to be synthesized in vivo with a molecular weight of about 48 kDa [Su et al., 1989] as had been predicted. However, more care should have been taken in determining exactly what contaminants may have been present in the 2PF antigen. In hindsight, perhaps 2PF protein should not have been used as the antigen. β-Galactosidase free L-SD could have been obtained, by digestion of the 3PF with collagenase and subsequent chromatographic manipulations, and used as antigen.

Figure 6: Western Analysis Detection of L-SD in Extracts

Two 10% SDS gels (I and II) were run simultaneously following the BIO-RAD manual and then either silver stained as in (I) or blotted onto nitrocellulose and probed with serum sample 00 (II) as described in Materials and Methods.

On both gels lane A was loaded with molecular weight markers, lanes B and C were loaded with affinity purified and collagenase-treated 3PF (7 and .7 µg respectively), and lane D was loaded with pure β-galactosidase purchased from Sigma (.5 µg). On gel II lane E was loaded with MEW28/pMES22 extract (1 µg), and lane F was loaded with MEW28 extract (1 µg).

The results given are from a single experiment typical of three.


I
II
A B C D
A B C D E F
97
66
43
31
21

PART 4. L-SD ACTIVITY IN VITRO AND IN VIVO

The availability of large amounts of highly purified 3PF made it possible for the first time to characterize purified L-SD. I began by determining the specific activity of L-SD in its fusion and non-fusion forms. This is important because one would expect that the fusion protein would have a lower activity due to the presence of the bulky β-galactosidase protein molecule, which could interfere with L-SD activity in many ways (see Discussion).

In fact, the fusion interfered with L-SD activity as seen in vivo [Su, 1991] but not in vitro, perhaps providing some clues as to the mechanism of L-SD activation.

4-1. The In Vitro Specific Activity of L-SD in its Fusion and Non-Fusion Forms

L-SD was assayed in preparations of the 3PF and 2PF proteins (Table 6, lines I and II). These were used to calculate the specific activity for L-SD on the basis of the molecular weight of the fusion and non-fusion forms of L-SD as described below.

The molecular weight of the 3PF protein is about 165 kDa per monomer (we assume that the hybrid protein is in a tetrameric form as is native β-galactosidase [Fowler and Zabin, 1983]). Using this molecular weight, the specific activity can be converted from U/mg of protein to U/mole of

hybrid protein monomer. Assuming that one L-SD molecule is attached to one β-galactosidase molecule, the U/mole of hybrid protein molecule would then be equivalent to U/mole of L-SD monomer. The molecular weight of the L-SD monomer is about 48 kDa [Su et al., 1989], thus the U/mole of L-SD monomer can be converted to U/mg of L-SD (Table 6, line III).

To see how much β-galactosidase interferes with L-SD I incubated a known amount of 3PF protein with collagenase and passed it through the FPLC (as in Fig. 2). I pooled the L-SD containing fractions in which L-SD is no longer covalently bound to β-galactosidase (Fig. 2, fractions 22 to 26, hereby termed FPLC L-SD) and determined the L-SD specific activity (Table 6, line IV), comparing the value with that calculated (Table 6, line III). It seems that the β-galactosidase interferes very little, if at all, and that even collagenase treatment followed by FPLC chromatography did not greatly decrease activity.

Iron and DTT are always required for L-SD activity, both in crude extracts and in purified preparations (Table 6, lines I, II and IV), without which L-SD activity can not be detected in vitro. This is important because iron and DTT are known to inactivate proteins by proteolytic degradation [Kim et al.,1985], thus the specific activity may be underestimated if an amount of L-SD has been degraded. It is interesting that a chemical treatment that is known to destroy proteins is required for L-SD activity.

TABLE 6: The Specific Activity of L-SD Protein Species

L-SD Species	L-SD With Fe,DTT (U/mg)	L-SD No Fe,DTT (U/mg)
I) Purified 3PF As Assayed	293	* NDT
II) Purified 2PF As Assayed	290	NDT
III) Separated L-SD And β-Gal As Calculated From (I)	1000	-
IV) FPLC L-SD As Assayed	855	NDT

The L-serine deaminase assay was performed as described in Materials and Methods using the standard L-SD assay, except that the enzyme was first treated with a low L-Serine concentration, 5 mM, for 15 minutes at 37°C with Fe and DTT in 50 mM HEPES buffer, pH 8 to activate the enzyme (see Materials and Methods). After this initial treatment, a high concentration of L-serine was added, 55 mM, and the assay continued for an additional 35 minutes at 37°C.

In line III, the specific activity was calculated from line I. In lines I and II, 3PF and 2PF proteins were first ATGA purified and then passed through the FPLC (as in Fig. 2) for further purification. In line IV, non-fusion L-SD (FPLC L-SD) was obtained from affinity purified 3PF that was collagenase treated and then passed through the FPLC as in Fig. 2, pooling the L-SD fractions with the majority of the L-SD.

The results are from a single experiment and are typical of three.

*NDT Stands for no activity detected above the absorbance observed in reactions with all components added except protein and all the components added except L-serine, which are typically between 10 and 30 klett readings at 540 nm. Assay values are typically between 100 and 400 klett readings at 540 nm.

4-2. Fusion Protein L-SD Activity *In Vivo*

Since L-SD was not inhibited by β-galactosidase *in vitro*, it was surprising that L-SD could not even be demonstrated *in vivo* [Su, 1991]. I repeated the *in vivo* experiments in somewhat more detail.

I compared L-SD activity of strains carrying the intact *sdaA* gene, the 2PF gene and the 3PF gene, all carried on pBR322, in a strain in which the chromosomal gene is nonfunctional due to an insertion of a chloramphenicol-resistant cassette in the *sdaA* gene (Table 7, line I). The native gene directed synthesis of a lot of active protein *in vivo* (Table 7, line II), whereas the fusion plasmids synthesized little or none, even under inducing conditions (Table 7, lines III and IV, with glycine and L-leucine). That is, although *in vitro* assays of purified protein (Table 6) and cell extracts (Table 7, column C: and Su, 1991) show that the cell synthesizes many L-SD molecules, under *in vivo* conditions none or very few of them are active. This is consistent with the finding that the *sdaA* plasmid allows a cell to grow on plates with L-serine as the sole carbon source, whereas the fusion plasmids do not [Su, 1991].

TABLE 7: In Vivo L-SD Activity

HOST	Plasmid	(A) No Gly No Leu (U)	(B) With Gly With Leu (U)	(C) In Vitro With Gly With Leu (U/mg)
I) MEW28	NONE	.002	.002	NDT
II) MEW28	pMES22	.350	1.000	4.5
III) MEW28	pMES27	.013	.023	4.8
IV) MEW28	pMES28	.005	.013	18.5

Cells were grown in glucose minimal media with (column B) and without (column A) glycine and L-leucine (300 µg/ml), subcultured and assayed as described in Materials and Methods. Antibiotic was added to plasmid-carrying cultures during both overnight growth and subculture as described in Materials and Methods.

The in vitro data obtained from the standard L-SD assay (column C) was derived using the extracts of column B cells. One unit of L-SD activity is the µmoles of pyruvate produced per 35 min at 37°C.

In the in vivo assay, one unit is defined as the µmoles of pyruvate produced by 0.1 ml of a 100-Klett unit suspension per 35 min at 37°C. Assay values for incubations lacking in substrate are subtracted from assay values of complete incubations. Only values above .005 units are cosidered significant activity [Newman et al., 1985b]

Experiment (I) was taken from reference [Su, 1991].

The data represents one experiment and is typical of at least three.

PART 5. THE INACTIVE FORMS OF L-SD

L-SD is originally transcribed and translated as an inactive protein [Su, 1991]. An unknown process converts the inactive protein to an active species *in vivo*. *In vitro* the activation process can be mimicked in crude extracts by incubating the extract with iron and DTT [Newman *et al.*, 1985a]. The same is true for purified protein preparations as shown here.

To better understand the activation process and hence the inactive form of L-SD, I performed *in vitro* experiments varying the concentration of the reducing agent, DTT. I then substituted DTT with β-mercaptoethanol, another reducing agent, in order to see if L-SD could still be activated.

Additional experiments were performed with protein that had been incubated at room temperature.

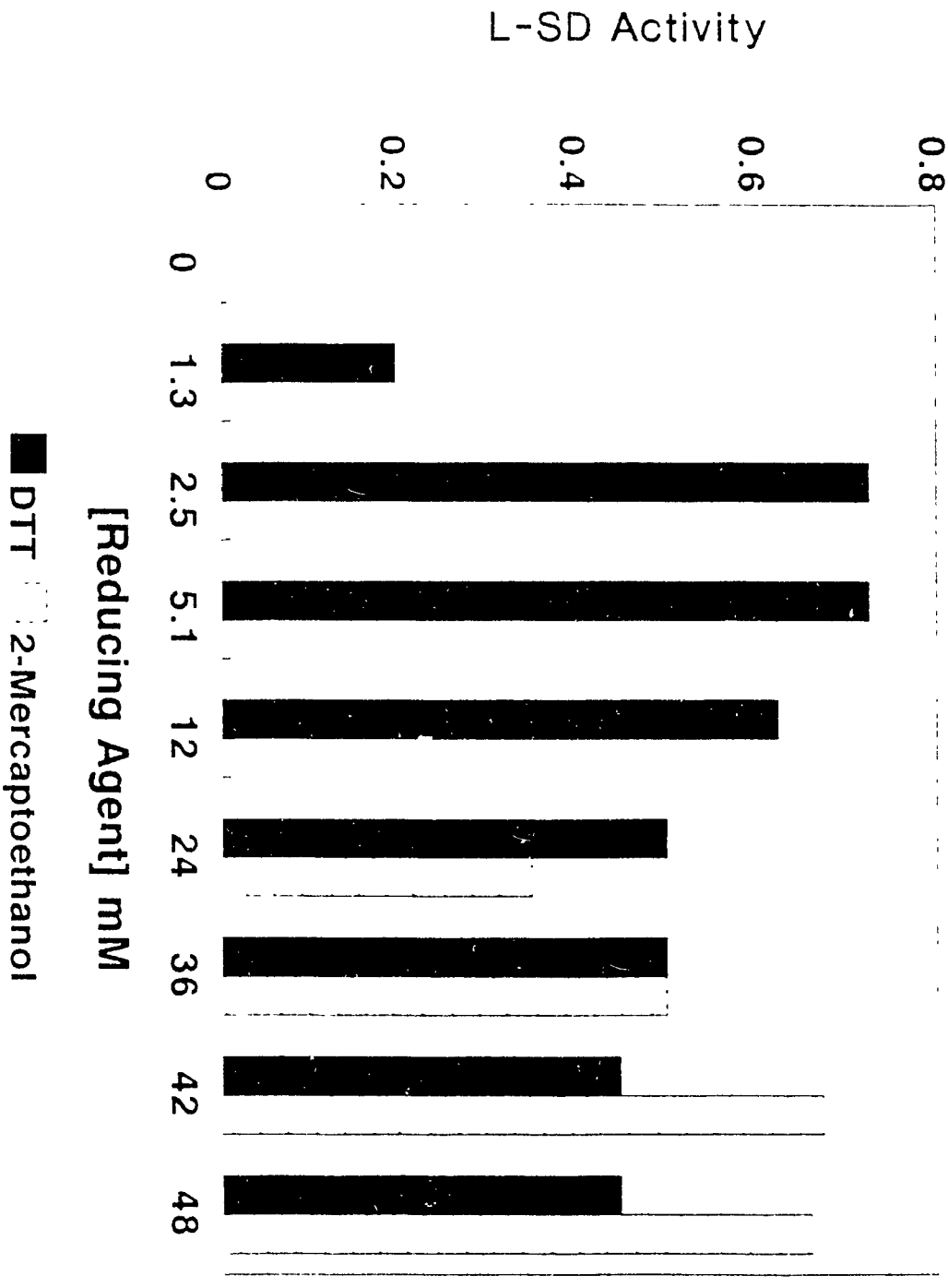
5-1. Activation of L-SD with DTT and β-Mercaptoethanol

DTT is a strong reducing agent. Could it be replaced by another reducing agent, such as β-mercaptoethanol? In order to determine this, I incubated protein with different concentrations of DTT and β-mercaptoethanol (Fig. 7) using a constant concentration of Fe (1.8mM $FeCl_3$) and L-serine (55 mM), and determined the amount of pyruvate formed after 35 min. at 37^0C.

Figure 7: The Activation of L-SD Using DTT or β-Mercaptoethanol

FPLC L-SD prepared as in Table 6, was assayed for L-SD using the standard L-SD assay at the DTT or β-mercaptoethanol concentrations indicated.

The data represents an average of 4 experiments.


L-SD Activity
0
0.2
0.4
0.6
0.8
0
1.3
2.5
5.1
12
24
36
42
48
[Reducing Agent] mM
DTT
2-Mercaptoethanol

Without reducing agent, no activity was detected. DTT was most effective at 2.5 to 5.1 mM although much of the activity could be seen with 10 times more DTT. With β-mercaptoethanol in place of DTT, very little activity was seen below 12 mM. At much higher concentrations, 42 mM (Fig. 7), β-mercaptoethanol was able to activate L-SD to the same extent as DTT did at the lower concentration of 2.5 mM. Whatever the structure of inactive L-SD may be and whatever the role reducing agents play in converting inactive L-SD to active L-SD, both DTT and β-mercaptoethanol can activate L-SD. However, a lower concentration of DTT than β-mercaptoethanol is needed to accomplish this.

5-2. High and Low DTT Forms of Inactive L-SD

L-SD has been thought to be very unstable [Newman and Kapoor, 1980]. However, it now seems that L-SD preparations lose the ability to be activated under normal activation conditions, but can be activated with altered activation conditions.

To show this, I compared the activity of various forms of L-SD from -70°C stored on ice, or incubated at room temperature for 3.5 hours. The protein incubated at room temperature showed little activity at the usual DTT

concentration of 4.45 mM (Table 8, line II), but at 10 times the normal level of DTT, activity was completely restored (Table 8, line III)

I also examined the effect of different concentrations of DTT on the activation of L-SD which had been incubated at room temperature. I found that the activation of this preparation required at least 25 mM DTT and was maximal at 45 mM (Table 9). At these higher DTT concentrations, L-SD activity is somewhat inhibited in freshly thawed protein (Fig. 7).

There seems to be two forms of inactive L-SD, the original one (IN#1) and a second which is obtained by simply allowing the protein to sit around at room temperature (IN#2). Both these forms can be activated, but at differing DTT concentrations. This raises questions as to what the characteristics of active L-SD may be, how L-SD may be activated, and how it may differ from inactive L-SD?

TABLE 8: The Activation of Freshly Thawed and Room Temperature Incubated L-SD Species

		L-SD Activity (U/µg)			
[DTT] (mM)	Pre Activation Incubation Time (min)	Crude Extract (A)	2PF (B)	3PF (C)	FPLC L-SD (D)
(I) 4.45	0	.013	.280	.300	.795
(II) 4.45	210	0	.005	.005	.005
(III) 45.0	210	.013	.275	.290	.801

Crude extracts (A), 2PF protein (B), 3PF protein (C) and FPLC L-SD were assayed for L-SD (I) and incubated for 210 min. prior to assay (II and III). L-SD activity was determined using the standard assay except that a higher DTT concentration was used in line III (45mM instead of 4.45mM).

The extract (A) was obtained from the L9 strain, grown in glucose minimal medium subcultured and harvested in late log phase, by sonication as described in Materials and Methods.

The 2PF (B), 3PF (C) FPLC L-SD (D) proteins were prepared as in Table 6.

All protein was stored in 20% glycerol at -70 C.

The data represents an average of 3 experiments.

TABLE 9: The Activation of Freshly Thawed L-SD with and without Pre-Incubation at Room Temperature

	DTT Concentration In mM	L-SD Activity (U/µg)	Room Temp. Incubation Time (min)
1)	4.45	.795	0
2)	4.45	.005	210
3)	10.0	.004	210
4)	15.0	.004	210
5)	20.0	.004	210
6)	25.0	.010	210
7)	30.0	.015	210
8)	35.0	.155	210
9)	40.0	.555	210
10)	45.0	.801	210
11)	50.0	.800	210

FPLC L-SD was assayed after it had been freshly thawed (1) or incubated for 210 min at room temperature as in Table 8 (2-11), except that differing DTT concentrations were used as noted.

The data represents an average of 3 experiments.

PART 6. THE STUDY OF ACTIVE L-SD USING AN LDH/NADH COUPLED ASSAY SYSTEM

The study of active L-SD is essential for the purposes of characterizing active L-SD, as well as to gain a better understanding of how L-SD is converted from an inactive to an active form.

To better understand active L-SD, I had to perform some initial experiments that would give me some basic, but important facts about L-SD. One of these would be an analysis of different buffering systems on L-SD activity and activation. Another would be the determination of the Km of L-SD and the nature of its interaction with various inhibitors. The stability of active L-SD is also an important question to address, especially in terms of further analysis of the active enzyme; if the active enzyme rapidly looses activity as originally proposed, one would have to look for stabilizing methods before studying it. In order to investigate these methods, it would be convenient to use a continuous assay system. The standard L-SD assay has been of great value but it is limited, it is a fixed time rather than continuous assay, and it has limited sensitivity in that small differences in activities cannot be easily demonstrated. I modified a coupled LDH/NADH assay and used it to study L-SD.

6-1. LDH/NADH Assay for L-SD

The LDH/NADH assay that I used is similar to that used by other investigators [Grabowski et al., 1991]. Lactate dehydrogenase (LDH) converts pyruvate to lactate with NADH being oxidized to NAD+ in the process. NADH absorbs at 340 nm while NAD+ does not. Thus by measuring the loss of absorbance over time, the enzymatic rate of LDH can be determined, and this reaction can be coupled with any reaction producing pyruvate. However, the use of the coupled assay for L-SD is complicated by the need to activate L-SD. In vitro L-SD can only be activated in the presence of iron and DTT and during this process iron precipitates out of solution, interfering with absorbance readings. To circumvent this problem I first activated L-SD with iron, DTT and low concentration L-serine at 37^{0}C for 15 min (as in Table 6). The presence of L-serine during the activation process is necessary for maximum activation [Newman et al., 1985a] and probably stabilizes the enzyme. I then centrifuged out the iron precipitate and added an aliquot of the incubation mix to a cuvette along with assay levels of L-serine, waited 2 minutes for the absorbance to stabilize, and then added LDH and NADH to allow the coupled reaction to proceed. Table 10 shows the readings obtained for the LDH/NADH assay. It is clear that not only was the rate of pyruvate formation with all components present (Table 10, line V) much higher than assay mixtures missing certain components (Table 10, lines I to IV), but the rate also increased with

TABLE 10: NADH/LDH Assay for L-SD Activity with Different Amounts of Protein

Contents Of Cuvette	Average Linear Rate (δAbs/30 Sec.) For 15 minutes 0.51µg Protein (A)	Average Linear Rate (δAbs/30 Seconds) For 10 minutes 1.0µg Protein (B)
I) Low Serine	.0005	.0006
II) No Protein	.001	.001
III) No NADH	0	0
IV) No LDH	0	0
V) Complete Assay	.044	.083

FPLC L-SD was prepared and activated as in Table 6, then assayed with the NADH/LDH assay in mixes with each component omitted in turn (lines I to IV) and with all assay components present (line V).

This data is taken from a single experiment and is typical of several.

TABLE 11: A Comparison of the Specific Activity of L-SD as Determined by the Standard L-SD Assay and the LDH/NADH Coupled Assay

	(A) Standard Assay	(B) LDH/NADH Coupled Assay
Specific Activity (U/µg)	.855	.704

(A) Standard assay activity data was taken from Table 6

(B) LDH/NADH coupled assay activity data was calculated from Table 10, using the formula $A=\varepsilon bC$ where:

$\varepsilon=6220/M$ cm for NADH

b=1 cm

C=[NADH]

A=Absorbance at 340 nm

increasing protein, 0.51µg (A) and 1.0µg (B) of protein gave rates of .044 and .083 δAbs/30sec respectively.

The coupled assay is not without its limitations. First, the assay is limited by the amount of NADH. For the experiments presented in Table 10, there was sufficient NADH for a linear assay of 15 minutes (A) and 10 minutes (B), after which most of the NADH is oxidized. The assay is also limited in that the rate immediately after activation, during centrifugation and subsequent 2 minutes, is not measured and could be higher than the rate eventually measured.

At this stage, it was clear that I had the coupled assay for L-SD working, but did it give accurate values for L-SD activity? Table 11 compares the specific activity of L-SD determined with the standard assay to that which was determined with the LDH/NADH coupled assay using the extinction coefficient for NADH. The values obtained are in reasonable agreement, so I proceeded to characterize active L-SD using the coupled assay system.

6-2. The Activity of L-SD in Different Buffers

It has previously been shown that L-SD is more stable in an organic buffer [Newman et al., 1985a]. However, only glycylglycine was studied.

In Table 12, data is shown for experiments in which other buffers were used. As an organic buffer HEPES was used, and as an inorganic buffer phosphate was used. It is clear that

TABLE 12: L-SD Activation and Activity in Differing Buffer Systems

Assay Conditions	L-SD Activity
(I) Activated In HEPES Assayed In HEPES	.706
(II) Activated In Phosphate Assayed In Phosphate	0
(III) Activated in HEPES Assayed in Phosphate	.700
(IV) Activated In Phosphate Assayed In HEPES	0

FPLC L-SD was prepared and activated as in Table 6. A 10µl aliquot of centrifuged activated protein mix was transfered into a cuvette, and L-SD measured with the NADH/LDH coupled assay as described in Table 10, in a total volume of 750µl. Activation and the assay were done in either HEPES or phosphate buffer, 50 mM pH 8 as indicated).

The L-SD activities were calculated as in Table 11. The data is an average of several experiments.

one can activate L-SD in HEPES and demonstrate activity in either HEPES or phosphate buffer (Table 12, I and III). However, L-SD could not be activated in phosphate buffer (Table 12, II and IV).

6-3. A Kinetic Analysis of Active L-SD

I next carried out a kinetic analysis on activated FPLC L-SD obtained as in Table 6. Fig. 8a presents the kinetic data of activated L-SD without any inhibitor. The apparent Km was found to be 25.5 mM and the apparent kcat 0.031 µmol pyruvate/µg protein/min. Previous investigators had determined the Km of L-SD as being 50 mM [Newman and Kapoor, 1980] in 50 mM glycylglycine, pH 8. My results indicate a lower Km, possibly due to the differing buffer conditions, glycylglycine may compete with L-serine for the L-SD active site. However, the Km for L-SD is still very large. Fig. 8b depicts a kinetic analysis of activated L-SD with 100 mM glycine added. The glycine appears to act as an inhibitor, raising the apparent Km to 40 mM. However, the apparent k cat remains relatively unchanged at 0.028 µmol pyruvate/µg protein/min. This data appears to indicate that glycine may be a competitive inhibitor of L-SD. However, the Km of L-SD (25.5 mM) being high, is not all that different from the inhibited enzyme (40 mM), or the originally determined uninhibited Km value of 50 mM, obliging me to conclude that

the only interesting observation that may be made from the kinetic analysis is that L-SD is an extremely high Km enzyme, as had been previously suggested [Newman and Kapoor, 1980].

6-4. The Stability of Active L-SD

While doing the kinetic analysis of L-SD I noticed that once activated, L-SD is stable in its active form. Table 13 shows a L-SD assay whose rate was followed over a period of four hours. Once activated L-SD remained active, and its activity remained linear, over the 4 hours of this assay. This is contrary to the previously held view that active L-SD is extremely unstable [Su, 1991].

Knowing that L-SD is stable once active, I proceeded to analyze differences between active and inactive L-SD in an effort to try and elucidate the mechanism of activation.

Figure 8: A Kinetic Analysis of Activated L-SD

FPLC L-SD protein was obtained and activated as in Table 6, then assayed with the DNPH/LDH coupled assay as in Table 10, in a 50mM HEPES pH 8 buffer, with the exception that the final L-serine concentration and post activation amino acid additions were varied as indicated in Fig. 8a and 8b.

The uninhibited Lineweaver Burke curve (1/rate as a function of 1/substrate concentration) of L-SD activity is depicted in Fig. 8a and with 100 mM glycine added (Fig. 8b) after activation.

The substrate concentration is expressed in mM and the rate is the change in absorbance (δAbs) measured at 340 nm every 30 seconds.

FIG. 8a

Km Of L-SD (No Inhibitor)

Michaelis Menten kinetics
Simple weighting

Variable	Value	Std. Err.
K cat	6.72543E-02	2.00949E-03
Km	2.54685E+01	2.47653E+00

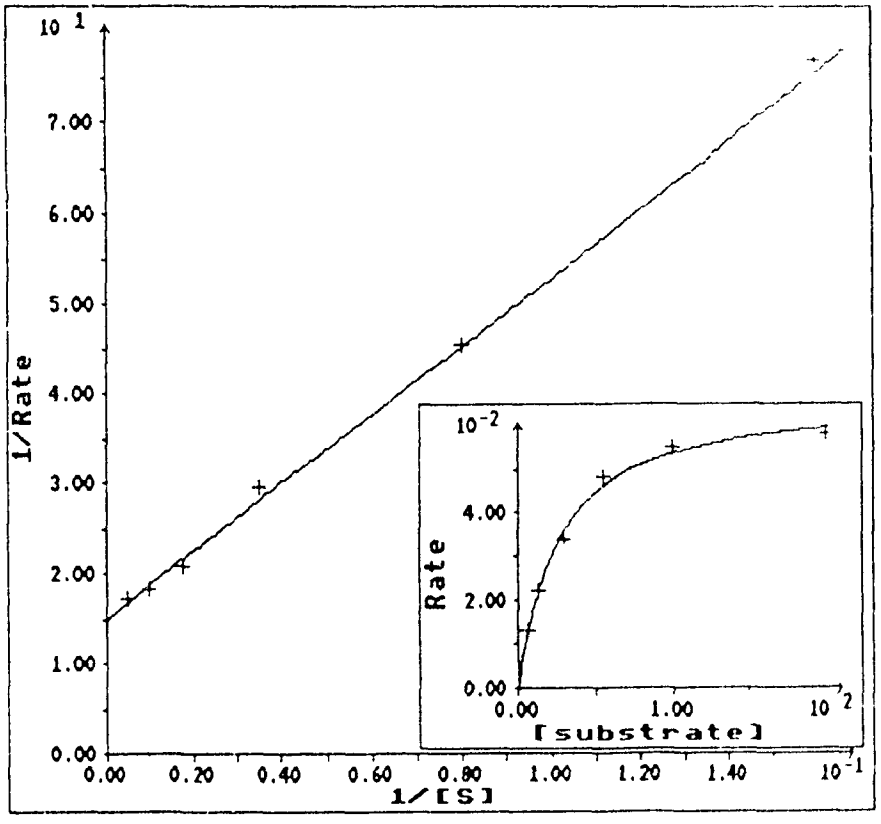


FIG. 8b

Km Of L-SD With 100mM Glycine As Inhibitor

Michaelis Menten kinetics
Simple weighting

Variable	Value	Std. Err.
V cat	6.03336E-02	9.22734E-04
Km	3.99617E+01	1.71870E+00

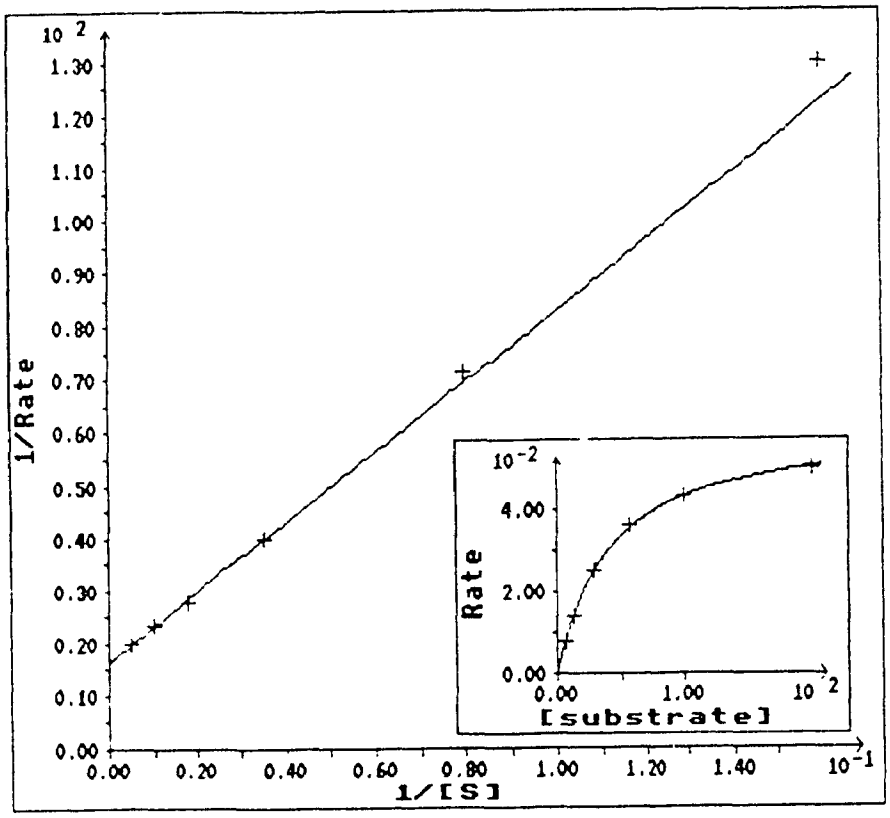


TABLE 13: The Stability of Active L-SD

Time	Rate After 1 hr	Rate After 2 hr	Rate After 3 hr	Rate After 4 hr
L-SD Act.	.010	.011	.011	.011

FPLC L-SD protein was obtained and activated as in Table 6, then assayed with the DNPH/LDH coupled assay (55 mM L-serine, .3 mM NADH and .33 µg/µl LDH) as in Table 10, in a HEPES pH 8 buffer. The enzymatic rate was followed in the same cuvette for a full 4 hours at 37°C and is shown here at 1 hour intervals.

The L-SD activities were calculated from the δAbs values using the extinction coefficient as shown for Table 11. The L-SD activity is given as U/µg prot/30sec.

These results are from a single experiment and are typical of three.

PART 7. THE COMPARISON OF INACTIVE AND ACTIVE L-SD

Based on the idea of serinolysis as proposed by Recsei [Recsei et al., 1983], it seemed that the in vitro activation of inactive L-SD may take place by cleavage with iron and DTT at the serine-serine bond of amino acids 243 and 244 [Su et al., 1989].

The following experiments were intended to demonstrate whether activation involves the cleavage of a peptide bond in vitro. Also, with the antibodies to 2PF, I attempted to observe the in vivo molecular weight of active L-SD for comparison with inactive enzyme. Since the activation process may also result in some type of conformational change, I compared the effect of limited exposure to trypsin on active and inactive protein.

7-1. FPLC Analysis of Inactive and Inactivated L-SD

In the previous section, I showed that L-SD is stable in an active form for many hours. However, throughout my investigation I observed that once activated, L-SD loses its activity during chromatography on a FPLC Superose 12 column equilibrated with 50 mM HEPES buffer, pH 8. This provides a rapid method for inactivating L-SD that has been activated.

The inactivation of once activated L-SD is shown in Fig. 9. In this experiment, collagenase treated 3PF was applied to the Superose 12 FPLC column before and after incubation with Fe, DTT and L-serine, and each fraction eluted was assayed.

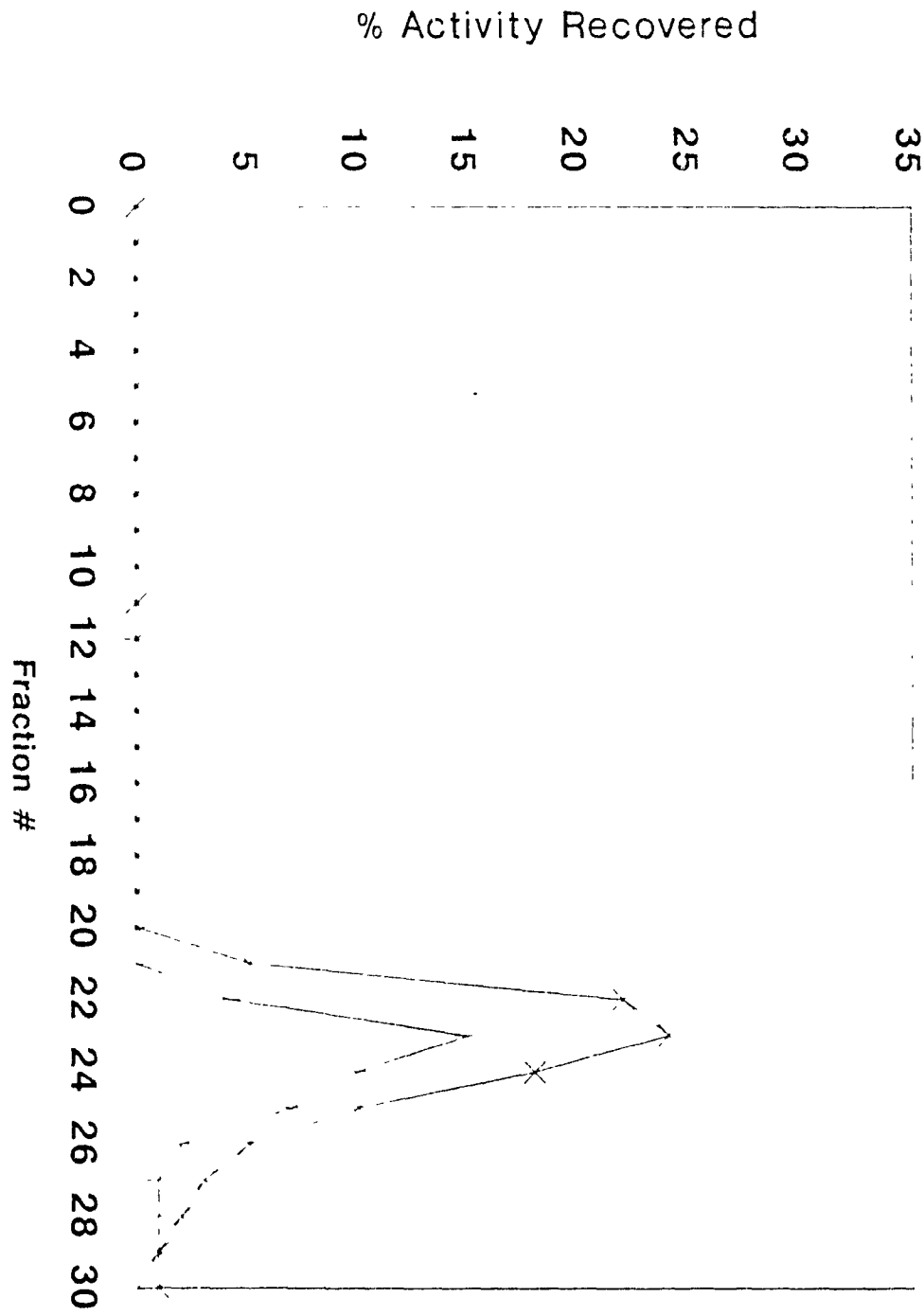
Figure 9: FPLC Analysis of Inactive and Activated L-SD Eluted in 50 mM HEPES, pH 8

Equal amounts of ATGA-purified collagenase treated 3PF, were left inactive (X) or were activated as in Table 6 (+) and eluted off a Superose 12 FPLC column equilibrated with 50 mM Hepes pH 8, at a flow rate of 0.5 ml/min, collecting fractions at 1 min intervals.

The fractions for both the inactive and activated protein had to be treated with iron and DTT (at standard L-SD assay concentrations) to measure the L-SD activity.

The results are given as the % activity recovered in each fraction compared to the total L-SD activity loaded on to the FPLC.

The inactive and activated L-SD were chromatographed separately. These results are from single experiments and are typical of several.


% Activity Recovered
0
5
10
15
20
25
30
35
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Fraction #

In both cases Fe and DTT was absolutely required to demonstrate enzyme activity in the fractions. Moreover, whether the L-SD preparation had been activated or not, the L-SD activity was found in the same FPLC fraction. This shows that there is no great change in molecular weight taking place on activation. However, some change in the enzyme does take place since I could regain only about 50% of the activity from the activated protein which had lost its activity on the FPLC.

7-2. Active L-SD off the FPLC

From Table 13, I know that L-SD remains active for a long period of time once it has been activated. Why then is L-SD inactivated when it is passed through the FPLC, a procedure that takes about half an hour? If serine was required to stabilize L-SD, perhaps the loss of its substrate as it passes through the FPLC resulted in the inactivation.

In fact, I was able to obtain active L-SD off the FPLC by repeating the Superose 12 chromatography using 50 mM HEPES, 50 mM L-serine, pH 8 as the column buffer (Fig. 10). The active enzyme peak (Fig. 10, fraction 23) recovered from the column eluted in the same fraction as the inactive enzyme peak (Fig. 9, fraction 23), suggesting that if activation involves proteolysis, the proteolytic products remain associated for activity. However, the elution profiles of the inactive and active enzymes are not identical. The inactive enzyme has a broader elution profile, indicating that there may be a

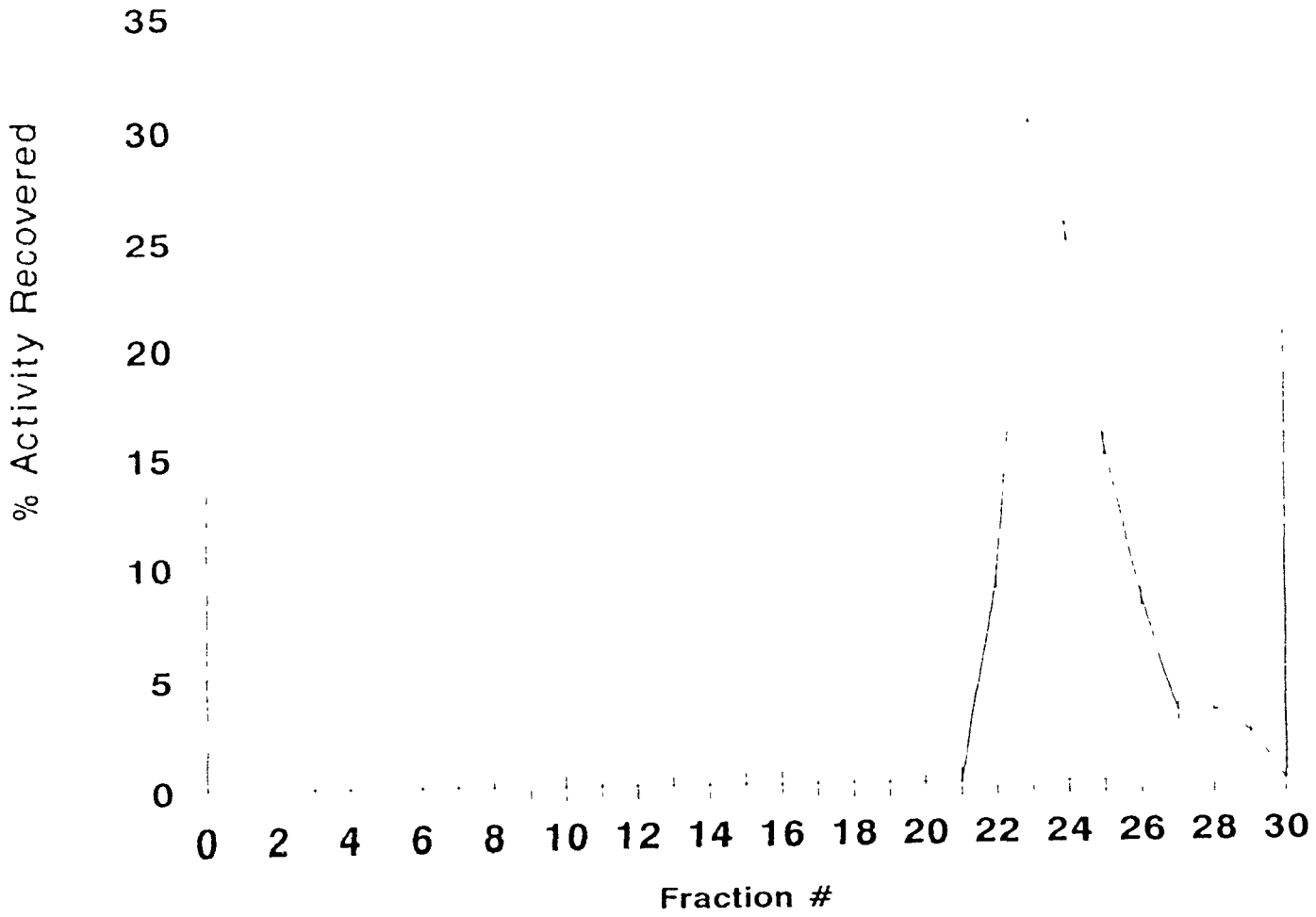
Figure 10: FPLC Analysis of Activated L-SD Eluted in 50 mM HEPES, 50 mM L-Serine, pH 8

ATGA-purified collagenase treated 3PF was activated as in Table 6 (+) and eluted off a Superose 12 FPLC column equilibrated with 50 mM Hepes, 50 mM L-serine pH 8, at a flow rate of 0.5 ml/min, collecting fractions at 1 min intervals.

The L-SD activity of the eluted fractions was measured without iron and DTT treatment.

The results are given as the % activity recovered in each fraction compared to the total L-SD activity loaded on to the FPLC.

The results are from single experiments and are typical of several.


% Activity Recovered
35
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Fraction #

difference between inactive and active L-SD. Indeed, even though the peak activities elute in the same fractions one could argue that the active enzyme elution profile, when compared to the inactive enzyme elution profile, has been shifted to a lower molecular weight. However, only SDS-PAGE experiments could determine if a cleavage accompanies activation of L-SD.

In addition, the fact that we were able to get active L-SD off the FPLC in the HEPES/Serine buffer indicates that substrate is not only necessary for optimum activation [Newman et al., 1985a], but is also necessary for maintaining a stable active protein.

7-3. SDS-PAGE Analysis of Activated L-SD

SDS-PAGE is different from FPLC chromatography in that denaturing conditions are used and thus non-covalently bonded peptides would be separated.

SDS-PAGE analysis of activated L-SD also indicated that the molecular weight of the enzyme did not change detectably as a result of activation. Figure 11 Gel 1, is an SDS-PAGE experiment in which samples of activated L-SD (lane D) and inactive L-SD (lane C) are compared. It is clear that there is no major change in the molecular weight of L-SD upon activation. Also, there are no lower molecular weight species apparent after activation. This data indicates that

Figure 11: SDS-PAGE of Inactive and Activated L-SD

10% SDS gels were run and stained as in Fig. 1.

In Gels 1 and 2, lane A was loaded with molecular weight markers, lane B was loaded with inactive ATGA-purified 3PF (9 µg) before treatment with collagenase, lane C was loaded with an equal quantity of lane B protein after treatment with collagenase as in Fig. 1 and lane D contains an equal quantity of lane C protein that has been activated as in Table 6.

In Gel 2 alone, lane E was loaded with an equal quantity of lane B protein (inactive 3PF) that had been activated as in Table 6.

Both gels in this figure represent a single experiment and are typical of several.

Fig. 11, Gel 1

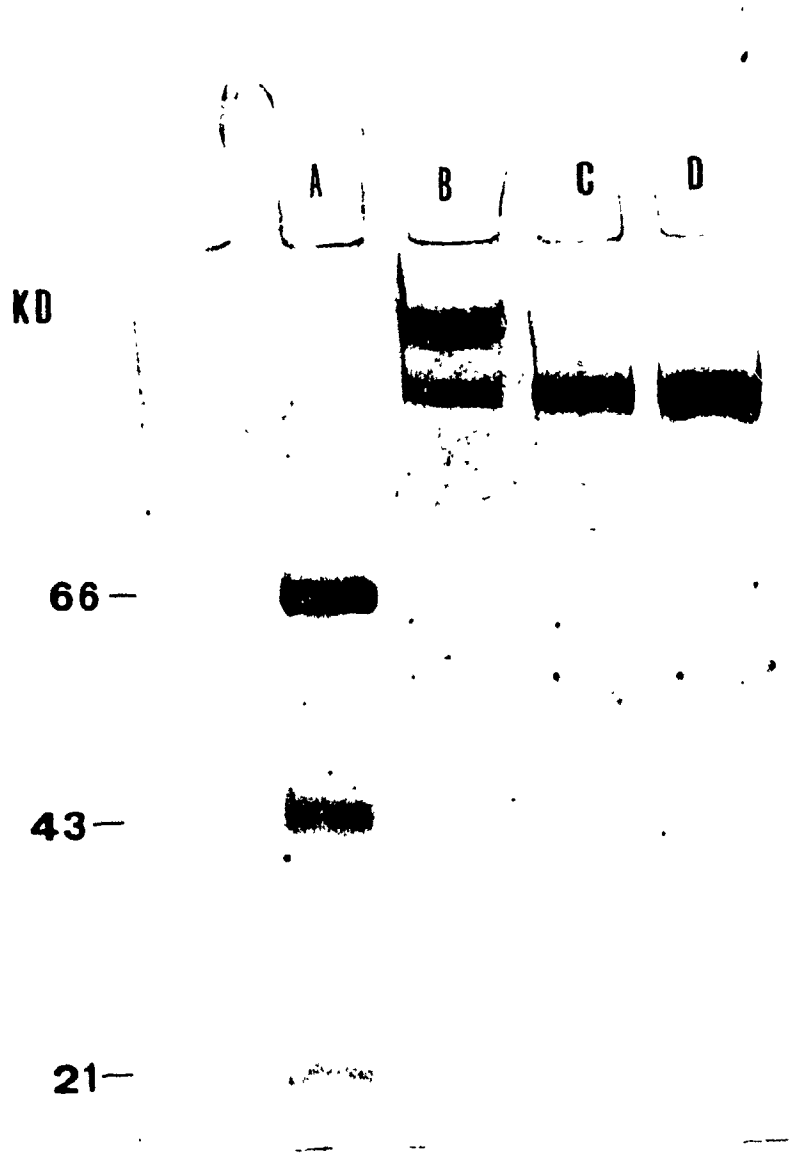
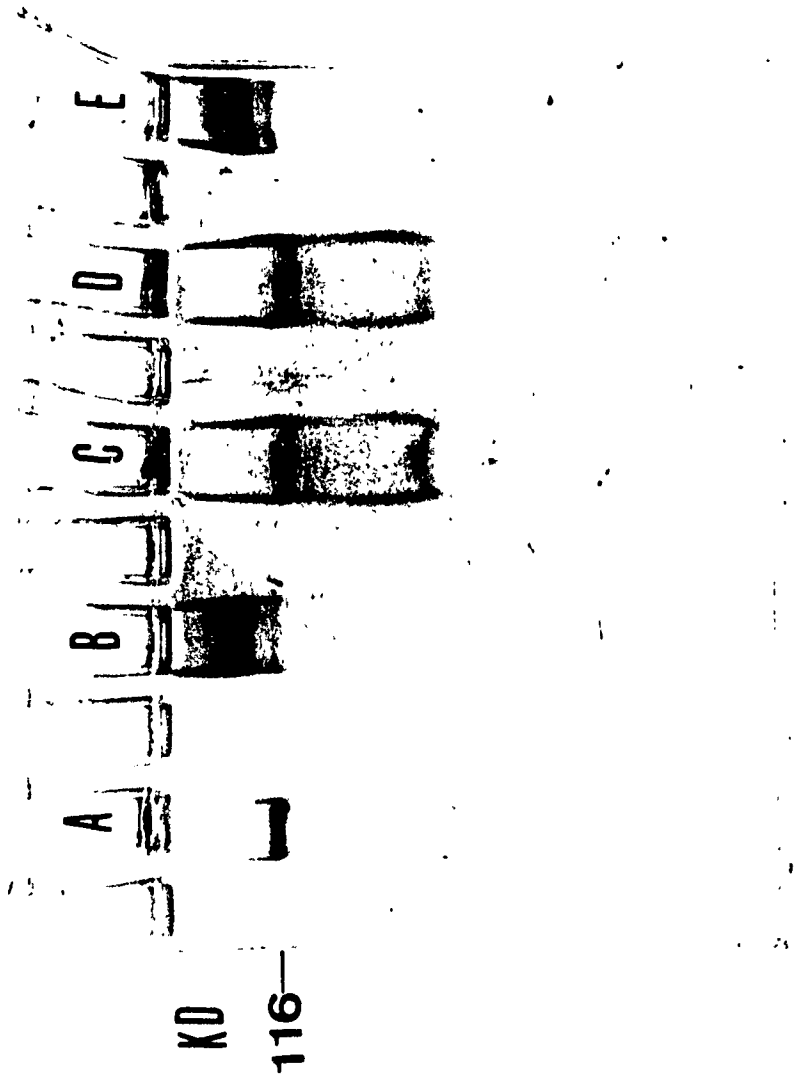


Fig. 11, Gel 2



activation either does not involve proteolysis or that proteolysis occurs so close to one of the ends that the corresponding molecular weight shift is not noticeable.

If, upon activation, proteolysis took place at the C-terminal of L-SD, then activating 3PF would result in the formation of separate L-SD and β-galactosidase molecules. In Figure 11 Gel 2, there was no separation of L-SD and β-galactosidase upon activation (lane D) of inactive 3PF (lane B). Therefore if a cleavage is involved in L-SD activation *in vitro*, then not only is it small but it also must be situated at the N-terminal of the protein.

7-4 The *In Vivo* Molecular Weight of Active L-SD

We do not know the *in vivo* molecular weight of active L-SD. If activation *in vitro* involves a cleavage, then activation *in vivo* may also involve a cleavage of some sort. Thereby using antibodies to determine the size of active L-SD *in vivo* is an important experiment.

For cells to grow on L-serine as the sole carbon source they require L-SD activity. Thus growing a strain that expresses the L-SD gene on L-serine as the sole carbon source ensures that active L-SD has been manufactured and utilized by the cells. One would therefore suppose that strain MEW28, which does not express L-SD or β-galactosidase when grown on NIV glucose [Su and Newman, 1991] would not have any L-SD protein or antibody reacting material, but strain

Figure 12: Western Blot Analysis of Active L-SD In Vivo

Duplicate 7.5% SDS gels were run as in Fig. 1, and either stained with Coomassie blue (Gel A) or blotted on to nitrocellulose (Blot B) and probed with antibodies as in Fig. 6.

Molecular weight markers were loaded in lanes 1 and 7. ATGA-purified 3PF (6 µg) was loaded in lane 2 and the same protein after collagenase treatment, as in Fig. 1, was loaded in lane 3. Extracts from MEW28/pMES22 grown on NIV glucose or NIV L-serine were loaded in lanes 4 and 5 respectively (10 µg), and MEW28 extract grown on NIV glucose was loaded in lane 6 (10 µg).

These results are from a single experiment and are typical of three.

1
2
3
4
5
6
7
BLOT B
KD
97
66
43
31
1
2
3
4
5
6
7
GEL A

MEW28/pMES22 should have high amounts of L-SD protein and activity, especially when grown on L-serine. Therefore, I probed with 2PF antibodies, a blot (Fig. 12, Blot B) of one of two duplicate SDS-gels (Fig. 12, Gel A) on which ATGA-purified inactive L-SD and extracts of MEW28 and MEW28/pMES22 (pMES22 synthesizes 25 kDa β-lactamase and 49 kDa L-SD) were chromatographed, to assess how much antibody reactive material there was and its molecular weight. The SDS-gel stained with Coomassie blue showed the high molecular weight band of ATGA-purified 3PF (lane 2) and a minor lower weight band which may correspond to β-galactosidase released from the fusion. The band corresponding to β-galactosidase band is seen (lane 3) after collagenase treatment, at a little higher molecular weight than the 97 kDa marker. However, the lower molecular weight L-SD band which should have also been produced after collagenase treatment is not observed, possibly due to the lower sensitivity of the Coomassie stain when compared to the silver stain used in Fig. 11.

The blot probed with antibodies showed a high molecular weight signal that may correspond to the 3PF in lane 2, as well as a minor signal at a lower molecular weight (between 66 and 43 kDa) that may be some L-SD released from the fusion. The L-SD after collagenase treatment can clearly be seen in lane 3. The L-SD producing strains grown on glucose and on L-serine, lanes 4 and 5 respectively, both show a signal coresponding to the same molecular weight as the L-SD signal

in lane 3. Lane 6 which contained extract from the non-L-SD-producing strain showed no L-SD signal. Of the two protein products of plasmid pMES22, only L-SD has the approximate molecular weight as the band observed in lanes 4 and 5. Therefore, there is no detectable molecular weight difference between the L-SD from strains grown on L-serine, on glucose, or purified L-SD. However, because of the initial shortcomings in the antibody characterization (Part 3 of the Results) there remains a possibility that the antibody signal may be against a purification contaminant. However if this were true I would have expected the MEW28 extract to have also shown a antibody signal and this was not the case.

7-5. A Trypsin Digestion Analysis of Activated and Inactive L-SD

Whether or not L-SD is activated by a cleavage, activation might involve a conformational change of some sort. Trypsin cuts proteins at the carboxyl side of lysine and arginine residues. However, susceptibility to tryptic cleavage depends upon the lysine and arginine residues being located on the protein so as to allow trypsin to act on them. Thus if a protein has more than one conformation, it may have differing susceptibilities to trypsin [Lei et al., 1979].

Figure 13 shows the effects of limited exposure to trypsin on activated and inactive L-SD, which appears as a

band between 43 and 66 kDa. When the protein is inactive no L-SD can be seen after a 10 minute exposure to trypsin (Fig. 13, Gel A, lane 9), but once the protein has been activated trypsin has little effect (Fig. 13, Gel A, lane 5). Similarly, at a higher concentration of trypsin all the inactive L-SD is completely digested after 1 minute (Fig. 13, Gel B, lane 6) and the active protein band is still present even after 5 minutes (Fig. 13, Gel B, lane 4), although the band has lost considerable intensity and lower molecular weight species are observed.

The different susceptibilities to trypsin suggest different conformations, and this suggests that Fe and DTT act to alter the conformation when activating the enzyme.

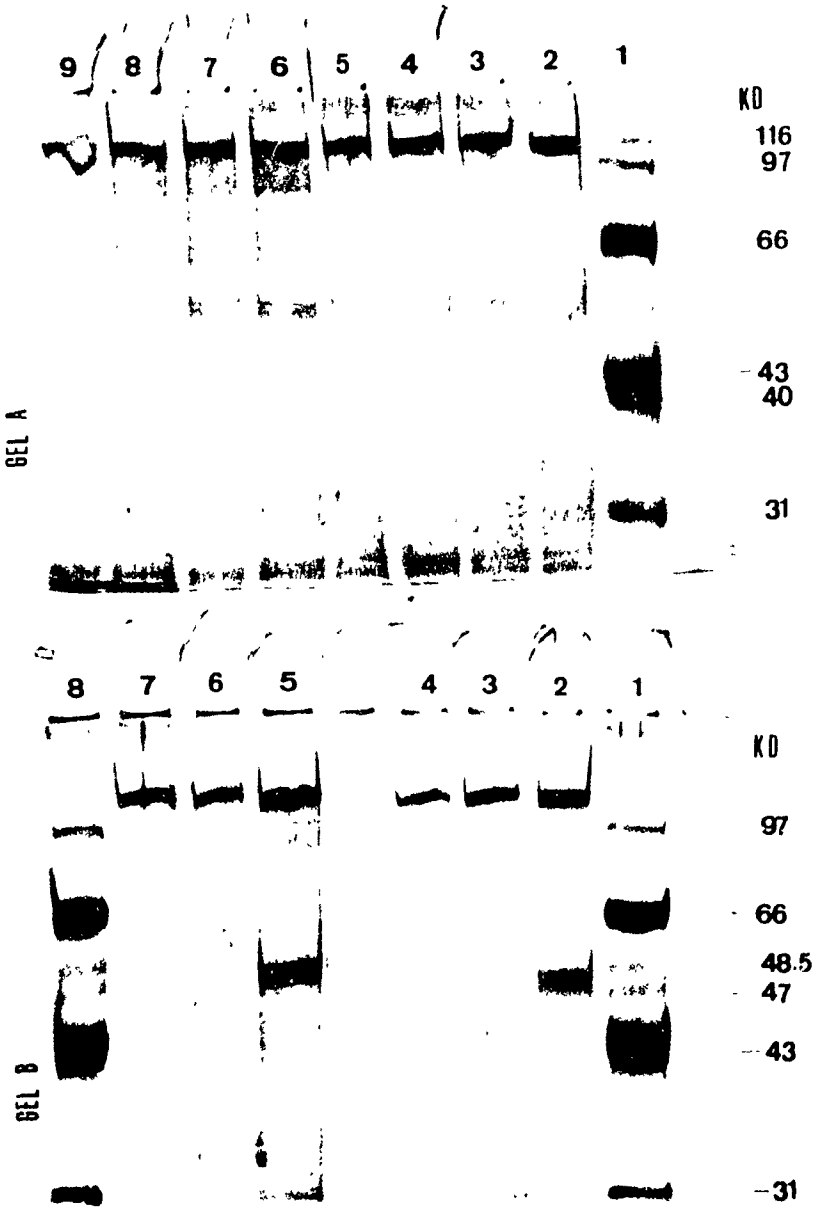
Figure 13: Effect of Limited Exposure to Trypsin of Inactive and Activated L-SD

7.5% SDS gels were run and stained as in Fig. 1.

In Gel A, lane 1 was loaded with molecular weight markers. ATGA purified 3PF (5 µg) was treated with collagenase as in Fig. 1, and either activated as in Table 6 (lane 2 to 5), or kept on ice (lanes 6 to 9), and then treated with trypsin (0.002 µg) for 0 (lanes 2 and 6), 2 (lanes 3 and 7), 5 (lanes 4 and 8) and 10 (lanes 5 and 9) minutes.

In Gel B, lanes 1 and 8 were loaded with molecular weight markers. Activated (lanes 2 to 4) and inactive (lanes 5 to 7) enzyme was prepared as above and treated with trypsin (0.02µg, 10 times the amount used in Gel A) for 0 (lanes 2 and 5), 1 (lanes 3 and 6) and 5 (lanes 4 and 7) minutes.

These results are from single experiments and are each typical of 3.


9
8
7
6
5
4
3
2
1
KD
116
97
66
43
40
31
GEL A
8
7
6
5
4
3
2
1
KD
97
66
48.5
47
43
31
GEL B

PART 8. CHAPERONE MEDIATED ACTIVATION OF L-SD *IN VIVO*

Though Fe and DTT are known to cleave proteins I was not able to show that cleavage was required, or even accompanied *in vitro* activation. The trypsin sensitivity experiments indicated that activation might be accompanied by a change of conformation. Then one might expect that Fe and DTT could activate L-SD by changing the conformation, and perhaps by changing disulfide bonds. If this were true then *in vivo* activation might also involve a conformational change, in this case an enzyme-catalyzed one.

Strain MEW28 has no *in vivo* or *in vitro* L-SD activity because it contains a null mutation in the chromosomal *sdaA* gene. In contrast, three mutant strains MEW128, MEW191C, and MEW84 (*in vivo* activation mutants) all carry the intact L-SD gene and have normal L-SD activity *in vitro* [Newman *et al.*, 1985a], but lack L-SD activity *in vivo* [Newman *et al.*, 1985b] as a result of mutations that map outside the L-SD gene. This suggested that L-SD is in an inactive form *in vivo* and requires activation by at least three different enzymes (each *in vivo* activation mutant lacking in one of these enzymes), a process that is mimicked chemically *in vitro* by Fe and DTT [Newman *et al.*, 1985a].

Chaperones are a family of proteins involved in the proper folding of polypeptides *in vivo*. In *E. coli*, the chaperone proteins DnaK and DnaJ are believed to first bind newly synthesized polypeptide chains as they emerge from

ribosomes, catalyzing the formation of a folded intermediate, which is then transferred to a GroES and GroEL chaperone protein complex by another chaperone named GrpE. GroES and GroEL are involved in the final folding process that gives rise to a properly folded protein [Langer et al., 1992].

L-SD may fold most easily into an inactive form in vivo, and remain in that form until the three activating enzymes, or more, act on it. If these activating enzymes are involved in L-SD folding then they may be similar to chaperone proteins. Therefore, if the in vivo activation mutants were supplied with an excess of chaperone proteins the misfolding of L-SD may be prevented, and perhaps give rise to L-SD activity.

To test this, Dr. Newman obtained plasmids carrying chaperone protein genes, i.e. groESL and dnaKJ (from Dr. Georgopoulos and Dr. Walker). These were transformed into the 3 in vivo activation mutants, the null mutant, and wild type strains. The strains were grown at 37°C and at 42°C with and without inducers of L-SD activity (glycine and leucine). No activity was seen in the 37°C growth conditions in any of the mutants with or without plasmids (data not shown). However one plasmid, pBR322 carying groES and groEL (pOF39), resulted in an increase in L-SD activity in all three in vivo activation mutants at 42°C with glycine and leucine (Table 14). The null mutant showed no activity under these conditions, as was expected since this strain synthesizes no functional L-SD. Similarly, wild type E. coli did not show any increase in L-SD activity, which is understandable since

TABLE 14: *In Vivo* L-SD Activity of Strains Grown at 42°C

Strain	Plasmid	L-SD Act. (mU)	
		NO Gly Leu	WITH Gly Leu
CU1008	NO PLASMID	30	165
CU1008	pBR322	30	165
CU1008	pOF39 (groESL)	30	168
CU1008	pBB1 (dnaKJ)	30	170
MEW28	NO PLASMID	5	5
MEW28	pBR322	5	5
MEW28	pOF39	5	5
MEW28	pBB1	5	5
MEW128	NO PLASMID	5	5
MEW128	pBR322	5	5
MEW128	pOF39	5	25
MEW128	pBB1	5	5
MEW191C	NO PLASMID	no growth	133
MEW191C	pBR322	no growth	129
MEW191C	pOF39	no growth	195
MEW191C	pBB1	no growth	135
MEW84	NO PLASMID	4	22
MEW84	pBR322	4	22
MEW84	pOF39	4	61
MEW84	pBB1	5	20

Cells were grown in glucose minimal medium, at 42°C, with and without glycine and leucine (300 µg/ml), subcultured and assayed as in Table 7. Assay values varied from 5% to 10% between experiments. These results were averaged from three experiments.

the strain should not be lacking in any of the *in vivo* activating machinery. The DnaKJ encoding plasmid (pBB1) had no effect on the *in vivo* activation mutants, indicating that perhaps the initial folding pathway of the *in vivo* activation mutants is not affected.

GroESL synthesis from plasmid pOF39 is under the control of a heat shock promoter. To test whether the increase at 42°C due to GroESL was the result of the heat shock promoter on the GroESL plasmid, or whether some other chromosomal gene regulation was involved, Dr. Newman obtained a plasmid with a lac operon promoter controling the groEL and groES genes from Dr. Sherman. Using IPTG (a lac operon inducer) to induce transcription, the results in Table 15 were duplicated (data not shown), but again only at 42°C with glycine and leucine in the growth media. Therefore the high temperature requirement is not due to the GroESL plasmid promoter, but is perhaps necessary for the expression of another chromosomal gene or genes.

IV DISCUSSION

The work in this thesis has been devoted to the study of the activation and activity of L-Serine Deaminase (L-SD#1), the gene product of sdaA, which is the enzyme responsible for L-serine deamination in E. coli K-12. L-SD is obtained in an inactive form both in vivo and in vitro, and must be activated enzymatically in vivo and chemically (with iron and DTT) in vitro by unknown mechanisms.

My investigation has proceeded through eight main phases, summed up briefly below, and will be discussed in more detail in the parts to follow.

Phase 1: L-SD#1 was purified in the 2PF and 3PF forms (L-SD fused to β-galactosidase and to β-galactosidase with an N-terminal collagen sequence respectively). The cleavage of the latter form with collagenase to yield separate β-galactosidase and L-SD protein molecules was demonstrated. The purification process also indicated that there may be conformational differences between the 2PF and 3PF proteins.

Phase 2: Proof that L-SD#1 is indeed encoded for by sdaA was obtained and the correct translational start site was identified through sequencing of the N-terminal amino acids of purified 2PF protein.

Phase 3: Purified fusion protein was used to raise antibodies for the purposes of identifying L-SD in bacterial extracts. The antibodies were also used in an attempt to elucidate the L-SD activation process.

Phase 4: The *in vitro* and *in vivo* activity of L-SD, both in fusion and non fusion protein forms, was investigated using standard L-SD assay techniques. The data indicated that when in the fusion protein form, β-galactosidase had little if any inhibitory effect on L-SD activity *in vitro*. However, neither the 2PF or 3PF proteins showed activity *in vivo*.

Phase 5: The activation of L-SD *in vitro* was studied with standard L-SD assay techniques and varying amounts of reducing agents. This avenue of study led to the identification of two different possible forms of inactive L-SD, IN#1 and IN#2. It was also showed that β-mercaptoethanol (ME) could substitute for DTT in the *in vitro* activation process.

Phase 6: The activity of L-SD was studied *in vitro* using a modified NADH/LDH coupled assay. The Km of L-SD was determined (25.5 mM for L-serine) and inhibition was studied. Additionally, the effect of different buffers on L-SD activation and activity was studied, as was the stability of active L-SD over long incubation periods.

Phase 7: The activation of L-SD *in vitro* was studied using gel filtration and SDS-PAGE and compared with *in vivo* activation using Western analysis. The results indicated primarily that there is no detectable cleavage, but that there may be a change in protein conformation involved in the activation process.

Phase 8: The *in vivo* activation of L-SD was studied using chaperone proteins, which are involved in protein folding. This line of study indicated that protein folding may play a role in L-SD activation *in vivo*.

PART 1. PURIFICATION OF L-SD

The goals of this research project were to characterize the activation and activity of L-SD#1, thought to be encoded by *sdaA* which has been cloned and sequenced [Su *et al.*, 1989]. L-SD had already been purified in the 2PF and 3PF forms. However, the purification had not been thoroughly quantitated. I therefore proceeded to quantitate the purification, during which I obtained evidence of a conformational difference between the 2PF and 3PF forms.

1-1. Purification of the 2PF and 3PF Proteins

H. Su constructed plasmids coding for the 2PF and 3PF proteins respectively [Su, 1991]. Both of these proteins were β-galactosidase fusion proteins, with the 3PF also containing a collagen sequence, which could be digested with collagenase, between the L-SD and β-galactosidase proteins (see Fig. i).

The purification of these proteins on an ATGA affinity column resulted in L-SD and β-galactosidase activities co-purifying (Tables 2, 3 and 4), as was expected if sdaA is the structural gene for L-SD and the sdaA and lacZ genes had been successfully fused. However, there were major differences in the purification results between the 2PF and 3PF.

In the 2PF purification, about 90% of the fusion protein adhered to the column and over 90% of that was easily eluted off the column with 20% D-lactose. In the case of the 3PF, only about 60% of the protein adhered to the column thus accounting for the lower overall % recovery of the 3PF when compared to the 2PF. This implies that a significant percentage of the 3PF as compared to the 2PF is not capable of binding to the ATGA column. Additionally, ATGA-adhered 3PF protein could only be successfully eluted using a 100 mM sodium borate solution pH 10.5 (Table 3) as was originally suggested in previous investigations [Ulman, 1984]. The sodium borate elution solution raises the pH to 10.5, and thus

elutes the 3PF by changing the charges of amino acids at the β-galactosidase active site, and throughout the whole protein.

What could account for such a difference in purification between the 2PF and 3PF species? The ATGA column binds β-galactosidase through active site binding [Ullmann, 1984]. The large percentage of 3PF that does not bind to the affinity column may have a conformation that does not bind substrate as effectively as does the 2PF. D-Lactose elutes β-galactosidase off the ATGA column by competing for the β-galactosidase active site. Therefore, the 3PF which adhered to the ATGA column but could not be eluted off by lactose may have a stronger active site binding than the 2PF, which had been easily eluted with 20% D-lactose.

That there appears to be more than one species of 3PF (weak and strong ATGA binding) is puzzling. The purification data revealed that the 3PF was synthesized at a high level (about four times the level of 2PF protein) due to the 3PF gene being on a high copy number plasmid and under the control of a heat shock promoter [Su, 1991]. In some cases, high levels of expression of fusion proteins in E. coli results in a fraction of the total pool of these proteins forming aggregates in the cytoplasm of the cell [Schein, 1989]. Therefore, a fraction of the 3PF may also be behaving similarly by forming a pool of aggregates that may be unable to bind to the ATGA column.

The only difference between the 2PF and 3PF that could account for the differences in the elution off the ATGA column is the 63 amino acid collagen sequence [Germino et al., 1984] contained in the 3PF. This collagen sequence may confer a different three dimensional structure to the 3PF as compared to the 2PF. Indeed, since it appears that the adhered 3PF is a stronger ATGA binding species, the 3PF structure may be more similar to the conformation of native β-galactosidase than the 2PF.

1-2. Separating L-SD and β-Galactosidase by Collagenase Digestion of the 3PF Protein

If sdaA does indeed code for L-SD then it should code for a protein of about 48 kDa [Su et al., 1989]. Treating purified 3PF with collagenase resulted in a cleavage of the protein to yield two smaller polypeptides. The larger of the two has the same molecular weight as β-galactosidase and the smaller of the two has a molecular weight of about 48.5 kDa (Fig. 1).

The shape of the 3PF protein is crucial for the purposes of digesting it with collagenase. β-Galactosidase is a tetramer in its native form, and thus it is easy to imagine a protein in which the collagen sequence would not be accessible to the collagenase for proteolysis. However, the fact that I am able to digest with collagenase implies that the collagen

sequence is accessible. Indeed, it has been reported that β-galactosidase fusion proteins have a perturbed structure when compared to the native wild type enzyme, the conformation being more open, particularly at the N-terminal domain of the protein [Fowler and Zabin, 1983].

Using gel filtration chromatography the 3PF protein eluted with both L-SD and β-galactosidase activities (Fig. 2). After treatment with collagenase the larger 3PF protein is replaced by two lower molecular weight species, the larger of the two having β-galactosidase activity and the smaller of the two having L-SD activity.

The gel filtration chromatography experiment was run under non-denaturing conditions. Therefore, a simple digestion of the collagen sequence of the 3PF does not ensure a separation of the L-SD and β-galactosidase proteins. They may very well have remained associated through non-covalent interactions. However, the fact that I was able to separate the two protein molecules, implies that there are no such interactions. The β-galactosidase and L-SD molecules may be well separated in space from one another by the linking collagen sequence, which is comprised of 60 amino acids from the triple helical region of chicken collagen [Germino and Bastia, 1984], and thus the two proteins would not associate after collagenase digestion. Additionally, both β-galactosidase and L-SD are soluble proteins and may not have regions of hydrophobicity that could interact with one another.

If the L-SD and ß-galactosidase molecules are separated from one another by an exposed collagen sequence, then there is the possibility that proteases could act on and digest the exposed collagen region preferentially.

1-3. Spontaneous Cleavage of the 3PF Protein

A close scrutiny of Fig. 1 shows that there is a slight band corresponding to β-galactosidase in the lane that had been loaded with non-collagenase treated 3PF (Fig. 1, lane B). There is no band corresponding to L-SD in this lane. However, when about twice as much protein was used, as in Fig. 11, Gel 1, it becomes apparent that purified 3PF preparations contain some already separated L-SD and β-galactosidase proteins (Fig. 11, Gel 1, lane B).

How can the above results be explained? There may be some protease contaminant in the purified 3PF preparations which may act on the exposed collagen sequence. While a sample of 3PF was digested with collagenase, an equivalent sample was incubated under identical conditions without collagenase (non-collagenase treated). A contaminating protease that co-purified may be able to act on the 3PF during this incubation. A second possibility is that a protease acted on the collagen sequence prior to purification. However, FPLC data (Fig. 2) suggests that L-SD and

ß-galactosidase do not interact with one another and would thus not be expected to co-purify if the covalent bonds linking them had already been broken. A third possibility is that in preparing the 3PF for gel electrophoresis by boiling it in SDS-loading buffer, the exposed collagen sequence was cleaved to some extent.

1-4. Problems Encountered with the Collagenase Treatment

Proteases such as trypsin and clostrapain are known to contaminate collagenase preparations and have been observed to complicate collagen sequence digestions in other tripartite fusion systems [Germino and Bastia, 1984]. The 3PF also appears to be somewhat affected by collagenase-contaminating protease activity. This activity is more readily observed in Western analysis experiments and will thus be discussed in detail in PART 3 of the Discussion, which is devoted to antibody experiments.

1-5. Why a Fusion Protein Purification Strategy?

Previous investigators had observed L-SD as an extremely unstable enzyme both in its inactive and active forms. As a result they were not able to purify L-SD using conventional purification strategies, hence the fusion protein purification of L-SD was employed.

With fusion protein purified L-SD a lot has been learned about L-SD, of which two points are important for future purification purposes. First, L-SD is not as unstable in vitro as had been thought in that the loss of enzyme observed by previous investigators [Newman and Kapoor, 1980] may have simply been the conversion of L-SD from IN#1 to IN#2 [see 5-2 of the Discussion]. Second, active L-SD is stabilized by L-serine [see 7-2 of the Discussion]. Knowing these two points now makes a conventional purification of L-SD more possible. I say this because there have been problems associated with the fusion protein purification of which the most difficult has been the continuous presence of ß-galactosidase, which has not only complicated the in vitro studies of L-SD activation but has also complicated the characterization and use of antibodies. Because of these problems I do not recommend that a fusion protein purification strategy be used if a enzyme has already been well characterized, even though this type of purification seems like a simple strategy.

PART 2. VERIFICATION OF THE L-SD AMINO ACID SEQUENCE

Although the purification of the fusion proteins gave strong, even conclusive evidence that the enzyme purified is the sdaA gene product, some questions may still remain. A straightforward way to address this problem was to perform an Edman degradation analysis on purified protein, thus determining its N-terminal amino acid sequence. Using this strategy, it was indeed verified that the protein I purified was the product of the sdaA gene. At the same time, the true sdaA translational start site was determined.

2-1. Edman Degradation of 2PF Protein

I had an Edman Degradation performed on the 2PF, whose N-terminal should correspond to the N-terminal predicted from the sdaA sequence.

The Edman data was in good agreement with the amino acid sequence predicted from the sdaA sequence (Fig. 3), except that the translation start site is 18 b.p. upstream of what had been originally proposed [Su et al., 1989].

2-2. Problems Encountered with the Edman Degradation

The Edman experiment was not without its problems. The first amino acid in the sequence could not be positively

identified. The GTG codon at the first position (Fig. 3, line 4) is recognized by formyl-Met-tRNA and should thus code for a methionine [Watson et al., 1987]. Also, the third amino acid which should be serine (Fig. 3, line 4) could not be positively identified. There was a low level of serine present in the sample that was sent for the Edman Degradation. As a result, serine peaks could not be elucidated. It is possible that our enzyme, being L-SD, would co-purify with some L-serine associated with it, thus accounting for the presence of the low levels of serine. However, the serine would then have to remain associated throughout the procedure used to prepare the 2PF for the Edman, which included SDS-PAGE and subsequent electroblotting. Also, despite my efforts to send as pure a sample as possible, there was the presence of a low level contaminating sequence which made sequencing past the first 8 amino acids difficult. SDS-PAGE experiments of my purified protein preparations almost always showed minor contaminant bands. If one of these contaminants was about the same molecular weight as the 2PF then it would be within the 2PF band, and I would have sent it along with the 2PF sample for sequencing.

I therefore conclude that sdaA codes for L-SD protein which is 454 amino acids long, instead of 448 as previously believed [Su et al., 1989], and has a molecular weight of about 48.8 kDa.

PART 3. ANTIBODIES TO L-SD

With a well defined purification of L-SD as proof that I was indeed working with L-SD from sdaA, we decided that the raising of antibodies to L-SD would be the next step in the investigation. Antibodies can play an important role in ascribing identities to bands on SDS-gels that would otherwise remain ambiguous.

3-1. Proving that Antibodies were Raised Against L-SD

Although Western blot and ELISA analyses show that I have most likely raised antibodies to L-SD, I have not shown this conclusively, for which additional characterization is necessary. One experiment is a Western blot analysis using the extracts of a ß-galactosidase deficient E. coli strain that has chromosomal sdaA expression (MEW1) and a sdaA minus derivative of this strain (MEW28). The MEW1 extract should yield antibody reactive material at about 50 kDa while the MEW28 extract should yield no antibody reactive material. In our lab G. Ambartsoumian is now studying L-SD expression in different mutants (8-1 of the Discussion) and in the course of his investigation has performed this experiment with the expected results [Ambartsoumian, personal communication]. Another experiment would be to probe blots of affinity purified collagenase treated 3PF and FPLC L-SD, both of which

should show about the same band intensity at 50 kDa but the former should have a more intense 116 kDa signal than the latter.

3-2. Protease Contamination of Collagenase

The collagenase purchased for use in our experiments still contained some protease contamination [Germino and Bastia, 1984; Boehringer Mannheim technical information]. This becomes apparent from an examination of the Western blot. Lane B of Fig. 6, I and II, contains a 31 kDa molecular weight species which may be the result of collagenase treatment. This band is also produced along with other minor signals in lane 3 of the Western blot B of Fig. 12 only after collagenase treatment, and is not visible in lane 2 of the same blot that was loaded with untreated 3PF. It seems likely that these post-collagenase treatment bands are the result of contaminating protease activity present in the collagenase.

The 31 kDa band does not appear in the extracts (Fig. 6, II lanes E and F; Fig. 12, B lanes 4, 5 and 6) and thus is likely to be a 3PF fragment. Also, the presence of this band is not consistent, i.e. it is not visible in Fig. 11, gels 1 and 2. Sometimes the band is observed after collagenase treatment sometimes it is not. Other investigators [Germino and Bastia, 1984] also encountered difficulties arising from protease contamination of collagenase and observed that slight

differences in collagenase digestion conditions altered the banding patterns that they ascribed to contaminating protease activity. In my case slight variations in the digestion conditions (e.g. the incubation time or temperature) may account for the variation in band formation. Indeed, the problems observed with the collagenase digestion is an argument against using a fusion protein purification strategy if other purification means are available.

3-3. Inhibition of L-SD Activity with Immune Serum

For additional supporting evidence of the existence of antibodies reactive against L-SD, I performed L-SD assays with and without serum present. The immune serum substantially inhibited L-SD activity (Table 5). There must be something in the immune serum, and lacking in the non-immune serum, that inhibits activity. Antibodies to L-SD are the most likely source of this inhibition. The L-SD/antibody interaction may block the active site or alter the protein in some fashion as to inhibit activity. On the other hand, it is also possible that the β-galactosidase antibodies may bind to the L-SD protein and affect L-SD activity. Also other antibodies or some unknown factor produced in the immune serum may be responsible for the inhibition.

The above experiment showed that activated L-SD was inhibited by immune serum. It might be interesting to perform

experiments that would indicate whether the immune serum inhibits L-SD activation as well.

PART 4. A COMPARISON OF L-SD ACTIVITY BETWEEN THE FUSION AND NON-FUSION FORMS

Up to this point the thesis has dealt with three different species of L-SD: the 2PF, 3PF and collagenase-treated 3PF, i.e. L-SD that is not bound to β-galactosidase. L-SD is obtained in an inactive form both in extracts and in the cell [Newman et al., 1985a; Newman et al., 1985b]. Activation of L-SD by iron and DTT was thought to involve a hydroxyl radical cleavage of inactive L-SD at a serine-serine bond in vitro [Newman et al., 1990; Su et al., 1989] and an enzyme-catalyzed process in vivo [Newman et al., 1985b]. It is possible that the three L-SD species may have differing levels of activity due either to their ability to be activated or their ability to deaminate L-serine once activated. The experiments discussed in this section indicate that their specific activities are identical in vitro but that the fusion proteins are inactive in vivo, a result that raises interesting questions as to the mechanism of the activation process.

4-1. *In Vivo* L-SD Inhibition by the Fusion Protein Species

H. Su found that *in vivo* there is no L-SD activity when L-SD is in the fusion protein form [Su, 1991]. I repeated and added to his experiments, obtaining the same results (Table 7). When an L-SD-deficient strain (*sdaA* interrupted by an insertion) is transformed with an *sdaA*-carrying plasmid, there is a significant amount of L-SD activity *in vivo* (Table 7, II). However, when this strain is transformed with plasmids coding for the 2PF and 3PF proteins, the *in vivo* L-SD activity is only slightly above background readings (Table 7, III and IV). Even when glycine and leucine are added to the growth media there is still only a small amount of *in vivo* activity in the 2PF and 3PF harbouring strains. Growth on glycine and leucine is known to increase L-SD activity as measured both *in vivo* and *in vitro* [Isenberg and Newman, 1974].

The β-galactosidase molecule might interfere with L-SD activity of the fusion proteins in several ways: (1) β-galactosidase could block substrate binding to L-SD; (2) β-galactosidase could prevent L-SD from attaining its active conformation by inhibiting activating enzymes from converting the inactive form to the active; (3) The presence of β-galactosidase could have resulted in the production of an inactive form of the enzyme that cannot be activated; (4) L-serine may be able to bind to L-SD but β-galactosidase could prevent L-SD from converting L-serine to pyruvate via steric

inhibition. Although near total inhibition was observed *in vivo*, *in vitro* experiments yielded opposite results.

4-2. L-SD Activity of Fusion Proteins is not Inhibited *In Vitro*

Having purified the 2PF and 3PF species, I proceeded to determine (Table 6) the *in vitro* specific activity of the 2PF, 3PF, and L-SD separated from the 3PF by collagenase treatment and FPLC chromatography (FPLC L-SD).

From the results in Table 6, it was clear that when L-SD is in the fusion protein form, there is no detectable hinderance in the activation and activity of L-SD *in vitro*. The specific activity that was determined for FPLC L-SD was .855 µmoles pyruvate/35 min/µg protein (Table 6, IV). For comparison purposes, the specific activity of L-Serine Dehydratase, a high Km enzyme, purified from *Lactobacillus fermentum* is .023 µmoles pyruvate/35 min/µg protein [Farias *et al.*, 1991], a value that is 38 times lower than that determined for L-SD.

4-3. Activation Mechanisms Suggested by the Activation Experiments

We do not known why the fusion protein is inactive *in vivo*. L-SD in the fusion proteins could be activated in

vitro. If this occurs by iron and DTT cutting and releasing active L-SD fragments [Newman et al., 1990], these active fragments would be free of any steric hindrance by fusion protein [Su, 1991].

Although this is sound reasoning there are other explanations not involving protein cleavage, i.e. it is possible that in vivo activating proteins simply can not reach L-SD when it is in the fusion protein form due to the bulky β-galactosidase procein. However in vitro, iron and DTT (or whatever activating agent they might form) being much smaller than protein molecules, may be able to reach L-SD in the fusion protein and change L-SD from an inactive to an active form with or without a cleavage being involved in the activation process. Further analysis is necessary to try and elucidate the activation process.

PART 5. INVESTIGATION OF THE ACTIVATION PROCESS USING VARYING AMOUNTS OF REDUCING AGENTS

A better understanding of the activation process of L-SD may be obtained by manipulating the activating agents iron and DTT. In previous work, various metals were substituted for iron. Copper ($CuCl_2$), aluminum ($AlK(SO_4)_2$) and zinc ($ZnSO_4$) were all found to be inhibitory [Newman et al., 1990] while magnesium ($MgSO_4$), manganese ($MnCl_2$), calcium ($CaSO_4$), potassium (K_2SO_4), nickel ($NiCl_2$) and sodium ($NaMoO_4$ and $NaWO_4$) had no effect on the enzyme. However, not much work had been

done with reducing agents. In this part, I discuss experiments that involve manipulating reducing agents and not the iron. These experiments led to the findings that ME can substitute for DTT and that there might be more than one form of inactive L-SD.

5-1. DTT and ME Both are Capable of Activating L-SD

From the data presented in Fig. 7 it is clear that DTT can be replaced as one of the activators of L-SD by ME, although much more ME is needed to achieve the same level of activation. A significant amount of activity is obtained at 2.5 mM using DTT as the reducing agent, while 42 mM of ME was required.

Why is DTT a better activator than ME? The most likely reason is that DTT is a much stronger reducing agent than ME [Burnes and Whitesides, 1990]. What if activating L-SD involves disulfide bond reduction? The L-SD amino acid sequence deduced from the sdaA DNA sequence contains 9 cysteines [Su et al., 1989]. These amino acids can form intramolecular disulfide bonds. The disulfide bonds can be cleaved by reducing agents such as DTT and ME, of which DTT should be more efficient.

Strong evidence already exists that reduction of the enzyme is involved in the activation of L-SD [Newman et al., 1990]. However, the reduction requirement is not the only

process necessary for activation. There is a requirement for oxygen along with both DTT and iron, suggesting that iron is not used as simple ferrous or ferric ion, but must cycle between the ferrous and ferric state, generating an oxidant which is also required for activation.

5-2. Two Different Inactive L-SD Species

From the experiments presented in Tables 8 and 9, it appears that there are two inactive L-SD species. The first inactive species is present in fresh crude extracts and purified protein preparations. After incubation at room temperature for a few hours, L-SD can no longer be activated at the usual DTT concentrations (4.45 mM, Table 8 II), but high activity can be attained at a larger DTT concentration (45 mM, Table 8 III) which is somewhat inhibitory to L-SD activity in freshly thawed preparations kept on ice (Fig. 7).

What happens to the enzyme as it incubates at room temperature? If reduction and conformational rearrangement is involved in enzyme activation, then L-SD oxidation is a simple explanation for what may be occurring. As L-SD (IN#1) sits at room temperature, it may become oxidized. This oxidized form of the enzyme may take on a different conformation (IN#2) through the formation of new disulfide bonds, or the shuffling of already existing disulfide bonds. The new conformation may not be as conducive towards reduction and would require a

higher amount of DTT for activation.

The finding of L-SD activity at the higher DTT concentration changes the previous conception of L-SD as an unstable enzyme [Newman and Kapoor, 1980]. It now seems that L-SD was not denatured or degraded in those experiments but may have been converted to a second form, IN#2.

Before studying the activation process further it became necessary to learn more about the characteristics of the active form of L-SD. Information on the active form of L-SD would not only be important in itself, but may also shed some light on the activation process.

PART 6. CHARACTERIZATION OF ACTIVE L-SD IN VITRO

To properly characterize L-SD activity, a coupled LDH/NADH assay was utilized. With this assay, I was able to study L-SD activation and activity in different buffers, perform a basic kinetic analysis of L-SD, and observe the stability of active L-SD.

6-1. The LDH/NADH Coupled Assay

A coupled LDH/NADH assay was modified for use in monitoring L-SD activity continuously. In order to determine if the coupled assay was useful, L-SD activity determined

using the coupled assay was compared with the L-SD activity determined using standard assay techniques.

The specific activity determined by the coupled assay is lower than the specific activity determined by the standard L-SD assay (Table 11), with an 18% difference between the results of the two assays. The fact that the coupled assay value is comparable to the standard assay value indicates that the coupled assay should be useful in studying L-SD activity. A comparison of the two assays leads me to believe that the coupled assay may be more reliable. The coupled assay differs mostly from the standard L-SD assay by the fate of the product of L-serine deamination, pyruvate. In the case of the standard assay, pyruvate builds up and may affect L-SD activity. The coupled assay, on the other hand, removes the pyruvate in a reaction with NADH to yield lactate.

6-2. L-SD Activation and Activity in Different Buffers

The coupled assay was first used to compare L-SD activation and activity conditions. From Table 12, it is clear that L-SD can retain an active form in either a HEPES or phosphate buffer system. However, it can be converted to its active form from its inactive form in the HEPES buffer but not in the phosphate buffer. Previous work indicates that a short lived species is required, possibly a hydroxyl radical, for L-SD activation [Newman et al., 1990]. Therefore a possible

explanation for the data of Table 12 is that in the phosphate buffer, the free radical necessary for activation can not be formed or is insufficiently stable. However, in the phosphate buffer, the enzyme may not be in an activatable conformation regardless of whether or not a chemical activating species is formed.

6-3. A Kinetic Analysis Of L-SD and Observations on Active L-SD Stability

The main use of the coupled assay was in performing kinetic experiments on purified L-SD. These experiments revealed that L-SD does not appear to deaminate L-serine efficiently.

In Fig. 8a the Km of L-SD is shown as 25.5 mM, an extremely high Km that leads to the question of whether L-serine is indeed the primary substrate of L-SD? Over many years, our laboratory has tried to find a better substrate for L-SD other than L-serine, as well as a different enzymic product without any success. Perhaps active L-SD does bind L-serine strongly, but the formation of pyruvate is not the primary function of the enzyme or it may simply be that L-SD binds L-serine strongly but the deamination reaction proceeds inefficiently because that is the way the enzyme was intended to work.

With antibodies to L-SD, an L-serine binding experiment could be performed to quantitatively determine L-serine binding to L-SD. Radiolabeled L-serine could be added at various concentrations to an incubation of activated L-SD and also to inactive L-SD to determine if binding takes place before activation. The L-SD would then be precipitated out of solution using the antibodies and binding of radioactive L-serine quantitated. The amount of binding could then also be compared to the pyruvate formation.

The characterisation of active L-SD led into a more detailed analysis of the activation process. The experiments that revealed active L-SD is stable in the presence of L-serine were useful in particular since they led to the isolation of active L-SD for comparison with inactive protein.

PART 7. A DETAILED ANALYSIS OF L-SD ACTIVATION

This section describes the characterization of the L-SD activation process. The experimental techniques, designed mainly to determine if the activation of L-SD involves a cleavage, involved visualising L-SD in its inactive and active forms. The results of these experiments, primarily indicating that there is no detectable cleavage but that there might be a conformational change of L-SD upon activation, will be reviewed.

7-1. Active and Inactive L-SD have no Detectable Molecular Weight Difference in their Non-Denatured States

In Fig. 9, inactive L-SD was observed to elute from an FPLC Superose 12 column (a gel filtration column that separates proteins by size) peaking in fraction 23. After L-SD was activated and run through the FPLC column, L-SD was no longer active in the eluted fractions. However, substantial activity was recovered in the eluate by treatment with iron and DTT, and the activity again peaked in fraction 23.

Since the L-SD activity was observed in equivalent fractions the species responsible for the activity must be the same, or nearly the same size. Also, the above experiments demonstrate that L-SD can be activated, with subsequent loss of activity that could again be regained by incubation with iron and DTT, a process implying L-SD may have the ability to interconvert between its inactive and active forms.

At this point there are two basic hypotheses regarding activation by proteolysis:

(1) Proteolysis by activation is not complete during the first activation, and subsequent activation after inactivation involves proteolysis of the remaining uncut protein.

(2) L-SD activation does not involve a cleavage and is reversible.

To further investigate the activation process, an experiment was performed (Fig. 10) in which active L-SD was

eluted off the FPLC gel filtration column with the activity again peaking in fraction 23, the same fraction as the inactive protein in the previous experiment. However, upon comparison of the two elution profiles one observes that the elution profile of active L-SD is somewhat sharper and more defined than the inactive protein. Therefore, although there is no detectable difference in the molecular weight of inactive and active protein there may be some difference between the two proteins that would account for the differing elution profiles.

Gel filtration chromatography separates proteins by their size by passing them through porous beads, i.e. the greater the size of the native protein the less it will be incorporated into the beads and thus the quicker the elution from the gel filtration column [Pharmacia, technical communication]. However, the shape of a protein can also play a role in the elution since proteins of equal molecular weights but differing conformations may interact differently with the pores in the column's beads. Therefore, the difference observed in the elution profile between inactive and active L-SD may be due to a conformational change.

Since active protein elutes off the FPLC column in the same fraction as inactive protein, if there is a cleavage of L-SD involved in the activation mechanism the fragments are not released but remain associated with the rest of the protein. Therefore the previous hypothesis, [Newman et al.,

1990], in which active fragments are liberated after proteolysis does not appear possible.

A cleavage of L-SD at the serine-serine bond at amino acids 243 and 244 [Su et al., 1989] can still be part of the activation mechanism. One can picture a non dissociating cleavage resulting in a conformational change which allows access to the active site and/or brings catalytic groups in the proper orientation. Evidence of this type of cleavage can be obtained by SDS-PAGE experiments.

7-2. Active L-SD is Stabilized by L-Serine

The isolation of active L-SD from the FPLC column in itself provides useful information about L-SD. I was able to isolate active L-SD by changing the buffer system from a 50 mM HEPES pH 8 buffer used in Fig. 9 to a 50 mM HEPES, 50 mM L-serine pH 8 buffer in Fig. 10. Dr. Judy Kornblatt suggested that active protein was inactivated during FPLC chromatography due to its substrate, which may be acting to stabilize an active conformation, being washed away. If the substrate, L-serine, is indeed necessary for maintaining an active form of L-SD, then equilibrating the column with buffer containing L-serine may allow for the active protein to retain its activity as it passes through the FPLC column, which is what appears to have happened.

Since I have been able to develop a technique for

obtaining active L-SD from the FPLC, some experiments can be conducted in the future. The Superose 12 column that I used does not completely separate β-galactosidase and L-SD (see Fig. 2) A different FPLC column can be used to isolate both active and inactive L-SD without any β-galactosidase impurity, then with these protein preparations, experiments could be performed to determine disulfide bond content. These experiments may indicate if disulfide bond reduction plays a role in L-SD activation.

7-3. No Detectable Molecular Weight Difference between Denatured Active and Inactive L-SD *In Vitro*

Polyacrylamide gel electrophoresis experiments were conducted in an effort to determine if activation involved a cleavage. The experiments appeared to indicate that active and inactive L-SD have the same denatured molecular weight (Fig. 11, Gel 1). The theory that L-SD is activated by a cleavage at the serine-serine bond of amino acids 243 and 244 [Su *et al.*, 1989] does not appear to be possible. Amino acids 243 and 244 are in the middle of the protein, thus if activation by cleavage at these amino acids was taking place then protein fragments would of been visible as lower molecular weight species in Figure 11, gel 1. However, there still exists the possibility of cleavages that would result in a small molecular weight change and would not be detected by

SDS-PAGE. How could a small cleavage be detected?

If the cleavage were at the N-terminal of L-SD, then an Edman degradation would show a difference between active and inactive protein. If the cleavage were at the C-terminal of L-SD then activating fusion protein (either 2PF or 3PF) would result in the cleavage of β-galactosidase from L-SD. Thus distinct β-galactosidase and L-SD proteins would be detectable after activating 3PF (much like the L-SD and β-galactosidase bands obtained after collagenase treatment of 3PF) on SDS-PAGE.

Figure 11, gel 2, indicates that there is no detectable cleavage of the 3PF upon activation. Therefore, if proteolysis is involved in the activation of L-SD, then it takes place near the N-terminal of the protein and is not detectable with SDS-PAGE.

What minimum size could a cleavage be and still be noticeable in an SDS-PAGE experiment? In Fig. 13, gel B, lane 1, I was able to observe a 1.5 kDa difference between molecular weight markers of 48.5 and 47 kDa. That is about a 14 amino acid difference in size. Therefore if there is a cleavage on the N-terminal of L-SD upon activation, I would estimate that it is less than 14 amino acids in size. There remains another less likely possibility, that activation does involve a cleavage, but so few protein molecules are activated that they are not detectable.

7-4. No Detectable Molecular Weight Difference between Denatured Active and Inactive L-SD *In Vivo*

With no evidence of activation *in vitro* involving a cleavage, I proceeded to study active L-SD *in vivo* to determine if a cleavage was involved in *in vivo* L-SD activation.

As shown in the Western Blot of Figure 12, active *in vivo* L-SD (Fig. 12, blot B, lane 5) appears to have the same molecular weight as inactive L-SD (Fig. 12, blot B, lane 3). If this experiment yielded lower molecular weight fragments, then the results would have been conclusive. However, the absence of lower molecular weight fragments could be due to the fact that active L-SD fragments, if they exist, may not be recognisable by the antibodies.

7-5. A Conformational Change may be Associated with L-SD Activation *In Vitro*

Having found no evidence for a cleavage associated activation, I performed experiments that would investigate the conformational states of inactive and activated L-SD. Limited proteolytic digestion was used to obtain data that indicated *E. coli* glutamine synthetase existed in different conformations [Lei *et al.*, 1978]. Using a similar approach, I performed tryptic digestion (Fig. 13) experiments that

indicated that active and inactive L-SD have differing susceptibilities to trypsin, and thus may have differing conformations.

PART 8. *IN VIVO* PROTEIN FOLDING AND L-SD ACTIVATION

If *in vitro* activation of L-SD by iron and DTT mimics activation *in vivo*, and I have found evidence for a conformational change *in vitro*, then one could postulate that a conformational change may also be involved in the *in vivo* activation process. With this in mind, *in vivo* experiments involving protein folding were performed, indicating that L-SD may indeed be activated *in vivo* by a folding mechanism, and discussed in this section.

8-1. Mechanisms of L-SD Activation by GroEL and GroES

The experiments presented in Table 14 imply that the chaperone proteins GroEL and GroES (GroEL/ES) somehow affect L-SD activity *in vivo*. How can these results be explained? Wild type *E. coli* has all the necessary components for *in vivo* L-SD activation, while the three *in vivo* activation mutant strains may each lack a different protein required for *in vivo* activation. That results were obtained using GroEL/ES is puzzling. The in vivo activation mutants are not GroES or GroEL mutations in that all the mutations map in different

locations on the E. coli chromosome from each other [Feng, 1990] and from the groEL/ES location [Fayet et al., 1986]. Therefore, GroEL/ES proteins should be present in the cytoplasm and thus the chaperone protein experiments would not be expected to yield results since all that I was doing in these experiments was increasing the levels of GroEL/ES.

Since results were obtained it implies that the in vivo activation mutants are deficient in GroEL and GroES type proteins, and are thus involved in the later stages of the protein folding pathway as are GroES and GroEL. DnaJ and DnaK proteins, which are involved in the initial protein folding stages, had no effect on activity.

The proteins missing in the mutant strains may be similar in structure and/or function to GroEL/ES in that they may be proteins involved in folding pathways. However, GroEL/ES may not be perfect substitutes for these proteins so that additional proteins may be required in order for GroEL/ES to have an effect, and these additional proteins might be made only if the cells are grown at 42°C with glycine and L-leucine. Indeed, the 42°C, glycine and L-leucine requirement also enhances the in vivo activation system of wild type E. coli [Newman et al., 1985b] suggesting that whatever is happening in the GroEL/ES experiments may be similar to the normal activation process.

It may also be that the GroEL/ES chaperone proteins and the in vivo activation system for L-SD may not function by activating L-SD but may actually prevent its inactivation in vivo. L-SD may not be conformationally stable [indeed, in vitro it seems to convert into a second inactive form (IN#2)]. L-SD may therefore be produced in vivo in an active form as are most other E. coli enzymes, but may be unstable and misfold into an inactive conformation, a process that may be hindered by the in vivo activation system or the chaperone proteins that have been introduced into the in vivo activation mutants.

The possibility also exists that GroEL/ES functions by somehow increasing the synthesis of L-SD in vivo, and thus increasing the observed activity. G. Ambartsoumian is now investigating this possibility with in vitro assays and Western blot analysis. However, if the mutants are truely deficient in L-SD activation machinery then increasing the level of L-SD should have little affect on activity because the additional enzyme molecules will not be activated. H. Su has performed experiments in which he introduced plasmid pMES22 (PBR322 carrying the sdaA gene) into the in vivo activation mutants. The presence of this high copy number plasmid increased the synthesis of L-SD in the in vivo mutants (more than 15 fold) as judged by in vitro L-SD assays but had little or no affect in the level of in vivo activity [Su, 1991]. H. Su performed these experiments at 37^{0}C and G.

Ambartsoumian has duplicated these results at 42°C in the presence of glycine and leucine for MEW84 and is obtaining data for the other strains [Ambartsoumian, personal communication].

8-2. The GroEL\ES Requirement for L-leucine may be Linked to Lrp

I have theorised that the requirement of glycine, L-leucine and 42°C for GroEL/ES to have an effect on L-SD activity may have to do with the formation of other gene products that may interact with the GroEL/ES proteins.

The temperature requirement may result in the formation of additional chaperone proteins that may function in conjunction with the GroEL/ES proteins, indeed additional chromosomal GroEL/ES will be synthesized at this higher temperature.

Another of these effectors, L-leucine, may act through the regulatory protein Lrp. The leucine-responsive regulatory protein, Lrp, is estimated to be involved in the regulation of 75 genes. Leucine is a major effector of the regulon, in that it affects Lrp regulation by binding to the protein [Newman et al., 1992]. The sdaA gene itself is regulated, in all or in part, by the Lrp protein [Lin et al., 1990]. It was suggested that the Lrp protein acts as a repressor which binds the upstream region of sdaA and that L-leucine releases this

repression [Su, 1991], increasing the transcription of sdaA in vivo. Similarly, it could be that L-leucine in my GroEL/ES experiments may be required to lift repression by Lrp from a gene whose product may be involved in an interaction with the GroEL/ES proteins. Perhaps glycine, like L-leucine, also acts on Lrp, or an Lrp-like DNA binding protein, to remove transcriptional inhibition, although glycine has not been shown to associate with Lrp.

Whatever the actual mechanism GroEL/ES uses to induce L-SD activity in vivo, it is likely to be extremely complicated especially when one considers the number of genes that might be regulated by L-leucine, glycine, and high temperature (42^{0}C). A great deal more work is required before anything concrete can be said. However, the fact that GroEL/ES are proteins involved in the proper folding of their substrates is supporting evidence that L-SD activity in vivo may involve protein folding pathways.

PART 9. SUMMARY

L-SD was purified in the 2PF and 3PF β-galactosidase fusion protein forms about 66 and 13 fold respectively. The digestion of the collagen sequence of the 3PF protein using collagenase to yield separate β-galactosidase and L-SD protein molecules was demonstrated. Purification data indicated that there may be conformational differences between the 2PF and

3PF proteins that could be the result of the collagen sequence possessed by the 3PF.

In vitro the fusion forms of L-SD were active but in vivo they were not. The lack of in vivo activity was likely due to the β-galactosidase part of the fusion proteins inhibiting the in vivo activation process.

Amino acid sequencing of purified 2PF protein was used to prove that L-SD (48.8 kDa) is indeed coded for by sdaA. The data also revealed that the translational start site of sdaA was 18 bp or 6 amino acids upstream of what was previously believed.

Purified 2PF protein was used to raise antibodies to L-SD which were used for identifying L-SD in bacterial extracts, and to indicate that there is no detectable molecular weight difference between active L-SD in vivo and inactive L-SD.

The activity of L-SD was studied in vitro using a modified LDH/NADH coupled assay. With the coupled assay the Km of L-SD was determined (25.5 mM for L-serine). Further kinetic analysis revealed that glycine is an inhibitor of L-SD activity. Additionally, L-SD could only be activated in a HEPES buffer and not a phosphate buffer, but demonstrated activity in both.

The activation of L-SD in vitro was thoroughly studied. Two different possible forms of inactive L-SD, IN#1 and IN#2 were identified, indicating that L-SD is not an unstable enzyme as once thought. It was also revealed that ME could

substitute for DTT in the in vitro activation process. It was shown that L-SD could cycle between an inactive and active form, and once activated L-SD was observed to remain active as long as L-serine was present. There was no detectable cleavage involved with, or even accompanying in vitro activation. However, there was data that indicated a change in protein conformation may be involved in the in vitro activation process.

The in vivo activation of L-SD was demonstrated using chaperone proteins (GroEL/ES), indicating that protein folding may play a role in L-SD activation in vivo.

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