This is to certify that the

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Molecular Cloning, cDNA Sequencing, Expression in E. Coli, Active Site Identification and Catalytic Mechanism of Pig Liver Thioltransferase

presented by

Yanfeng Yang

has been accepted towards fulfillment
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W. W. Wells
Major professor

Date 10/29/90

MOLECULAR CLONING, cDNA SEQUENCING, EXPRESSION IN *E. COLI*, ACTIVE SITE IDENTIFICATION AND CATALYTIC MECHANISM OF PIG LIVER THIOLTRANSFERASE

by

Yanfeng Yang

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for the degree of

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Department of Biochemistry

1990

ABSTRACT

MOLECULAR CLONING, cDNA SEQUENCING, EXPRESSION IN E. COLI, ACTIVE SITE IDENTIFICATION AND CATALYTIC MECHANISM OF PIG LIVER THIOLTRANSFERASE

by

Yanfeng Yang

Thioltransferase, also known as glutaredoxin, is a cytosolic enzyme that catalyzes the reduction of disulfides of various molecular weights in the presence of a monothiol, especially glutathione (GSH). Studies have led to the suggestion that thioltransferase may play an important role in the regulation of protein function, for example, enzyme activity, by altering the thiol or disulfide status through thiol-disulfide exchange reactions. Pig liver thioltransferase (PLTT) consists of 105 amino acids with a molecular weight of 11,740, and cysteine-22 has been proposed to be the active site of the enzyme.

A thioltransferase cDNA clone was obtained by screening a pig liver cDNA library in λ-gt11 with polyclonal antibodies against PLTT. For verification, this isolated cDNA was hybridized with three oligonucleotides synthesized according to the known amino acid sequence of PLTT. The confirmed PLTT cDNA was subcloned into M13mp18 at the EcoRI site and sequenced using the dideoxy chain-termination method. The amino acid sequence deduced from the cDNA sequence agreed exactly with that determined directly, except that the N-terminus should be N-acetylalanine followed by glutamine rather than the reverse as originally reported. This cDNA, with an introduced NcoI site at its initiation codon region, was cloned into an expression vector, pKK233-2, between the NcoI and HindIII sites and expressed in E. coli JM105 at a

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high-level (8% of total soluble protein). The recombinant enzyme was identical to the native enzyme in amino acid composition, thiol-disulfide exchange activity, and kinetic properties except no N-acetylation occurred at its N-terminus (alanine).

The speculated active site, Cys^{22}-Pro^{23}-Phe^{24}-Cys^{25}-Arg^{26}-Lys^{27}, of PLTT was directly tested by exchanging Cys^{22} with Ser^{22}, Cys^{25} with Ser^{25} or Ala^{25}, Arg^{26} with Val^{26} and Lys^{27} with Gln^{27}. Comparison of these mutants with the wild-type enzyme revealed that Cys^{22} is the catalytic site and its low pK_a (3.8) is facilitated by Arg^{26}. Not expected was the finding that the Ser^{25} mutant had an increased rather than decreased activity, indicating that the formation of an intramolecular disulfide between Cys^{22} and Cys^{25} is not required for the enzyme catalytic mechanism of mutants at position 25. However, the Ala^{25} mutant lost 91% of the wild-type enzyme activity, suggesting that an amino acid with a more polar side chain than a methyl group, such as $-CH_2OH$ or $-CH_2SH$, is required at position 25. The roles of Lys^{27} and the second pair of cysteines, Cys^{78} and Cys^{82}, were also investigated. Alternative catalytic mechanisms for thioltransferase were proposed according to radioactive labeling and kinetic studies of these mutant enzymes.

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To

my mother and father

as well as

my wife and son

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I also acknowledge Dr. Arnold Revzin and the people in his laboratory for offering their assistance and experience for my molecular cloning work.

Finally, my deepest thanks is for my wife, Jianli, and my son, Dan, whose love and full cooperation are always the spiritual source of my working energy.

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Cys-S

DHA

DMSO

DTT

ETT

ETT-am

β-gal

X-gal

GIT

HED

IAA

IAM

IPTG

LB-med

PDI

PLTT

PMSF

PPO

RRase

SDS-PAG

TBST

LIST OF ABBREVIATIONS

$Cys-SO_3^-$	S-sulfocysteine
DHA	dehydroascorbic acid
DMSO	dimethylsulfoxide
DTT	dithiothreitol
ETT	expressed pig liver thioltransferase
ETT-amino acid'	expressed mutant thioltransferase with the mutant amino acid's position
β-gal	β-galactosidase
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
GIT	GSH-insulin transhydrogenase
HED	hydroxyethyl disulfide
IAA	iodoacetic acid
IAM	iodoacetamide
IPTG	isopropyl-β-D-thiogalactopyranoside
LB-medium	Luria-Bertani medium
PDI	protein disulfide isomerase
PLTT	pig liver thioltransferase
PMSF	phenylmethylsulfonyl fluoride
PPO	2,5-diphenyloxazole
RRase	ribonucleotide reductase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST	Tris buffered saline with 0.05% Tween-20

CHAPTER I

LITERATURE REVIEW

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It is well known in recent years that the thiol-disulfide equilibrium directly or indirectly exerts an influence on various cellular processes, such as enzyme regulation (1-14), ribonucleotide reduction (15,16), protein synthesis and modification (17-19), calcium metabolism (20), lipid peroxidation (21,22), and hormone metabolism (23,24). In most cases, if not all, the modulation of the above activities is fulfilled by modification of thiols or disulfide of the enzymes through reversible oxidoreduction. For example, fructose 1,6-bisphosphatase and phosphofructokinase, two key enzymes in carbohydrate metabolism, are regulated by the thiol/disulfide ratio, _in vitro_, when treated with the disulfide cystamine. The former is stimulated whereas the latter is inhibited (25). In principle, accessible enzyme thiols are capable of forming protein-mixed disulfide or intramolecular disulfide, _in vivo_, by reaction with cellular disulfides. Such oxidation could result in either increasing, decreasing or not affecting enzyme activity, some examples of each are listed in table I.

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine), a tripeptide, which was found and originally named "philothion" one century ago by J. de Rey-Pailhade (26), is the major cellular thiol/disulfide redox buffer and undergoes thiol (GSH)-disulfide (GSSG) interchanges, _in vivo_ (27). The GSH/GSSG ratio in cells is high with a range between 100 to 300, and the ratio may be changed by hormone induction, as reported (28). In such a strongly reduced environment, the ratio of spontaneous thiol/disulfide exchange between protein thiols and low molecular-weight disulfide is considerably slow at physiological pH and is non-specific (29). Accordingly, there is likely to be a class of enzymes that

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Table I

Enzyme activity modified by disulfide treatment

Enzymes	Sources	effect	References
Glycogen phosphorylase phosphatase	rabbit liver	inactivation	1
Glycogen Synthase D	rabbit liver	inactivation	2
Phosphofructokinase	rabbit skeletal muscle	inactivation	3
Fructose 1,6-bisphosphatase	rabbit liver	activation	4
Hexokinase	bovine brain	inactivation	5
Glucose-6-phosphatase	rabbit liver	no effect	6
Pyruvate kinase	rat liver	inactivation	7
Glucose-6-phosphate dehydrogenase	rat liver, heart	activation	9
Acetyl-CoA hydrolase	rat pineal	activation	13
Acid phosphatase	spinach	activation	14

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catalyze these reactions in vivo. In the past couple of decades, several enzymes with general thiol-disulfide interconversion activity have been investigated in detail.

Protein-Disulfide Isomerase and Thiol:Protein Disulfide Oxidoreductase

Reduced unfolded proteins can spontaneously reoxidize and refold to form their native functional disulfide conformation. The nonenzymatic process, however, is not efficient (30). During a search for a cellular catalyst for this process, protein disulfide isomerase (PDI, EC 5.3.4.1.) was independently identified by the research groups of Anfinsen (31) and Straub (32). PDI is thought to catalyze thiol/disulfide exchange reactions involved in the posttranslational formation of disulfide bonds. It also can rearrange the disulfide bonds of a denatured "scrambled" protein (e.g. ribonuclease) to the native form, and in the presence of reductant thiols, this reaction was used as an assay method for PDI activity (33). PDI is widely distributed in animal and plant tissues, especially rich in those cells with major roles in synthesis of disulfide-bond containing proteins, for instance, mammalian liver, pancreas, lymphoid, chick embryo, and wheat endosperm, which implies a correlation between the level of PDI and the extent of synthesis of secretory proteins (19). PDI is one of the most abundant proteins in microsomes and its subcellular location is found in the lumen of both rough and smooth endoplasmic reticulum and loosely associated with the surface of the ER membrane (34). The distribution and subcellular location of PDI are highly correlated with its speculated function, catalyzing the formation of disulfide bonds of newly synthesized proteins in the lumen of the endoplasmic reticulum.

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Since 1960s, PDI has been purified to homogeneity and characterized from calf (35,36), rat (37,38), and mouse (39). This enzyme appears to be a homodimer with a molecular weight of 2 x 57,000 and an acidic pI of 4.2 (36). The primary structure of PDI was first determined by nucleotide sequencing of a cDNA for rat pancreas PDI (40) The deduced amino acid sequence consists of 508 amino acids (Mr 56,783) and has two internal homologous regions which are highly comparable to the active site sequence, Trp-Cys-Gly-Pro-Cys-Lys, of E. coli thioredoxin, a small cytosolic enzyme of 12 kDa. Both PDI regions contain the postulated active site with the sequence, Trp-Cys-Gly-His-Cys-Lys, confirming that it should have the ability to catalyze thiol-disulfide exchange reactions, a characteristic of thioredoxin (40). The partial sequence of a cDNA for human liver PDI was also determined and its deduced amino acid sequence showed 94% sequence homology to the rat enzyme, and like the rat enzyme, two sequences with similar identities to that of the E. coli thioredoxin active site were found (41). When the sequences were reported, PDI was compared with numerous other proteins, and several extended roles for PDI were suggested.

Prolyl-4-hydroxylase (EC 1.14.11.2), a tetrameric enzyme ($\alpha_2\beta_2$) with a molecular weight of 250,000, catalyses the formation of 4-hydroxy proline in collagen and other proteins with collagen-like amino acid sequences by the hydroxylation of proline residues in peptide linkages (42). The ß-subunit of this enzyme has been identified as PDI. Comparison of the cDNA sequences of the human hydroxylase ß-subunit and that of rat PDI shows that the degree of homology was 84% and 94% at the level of nucleotide sequence and the deduced amino acid sequence, respectively. Southern blot analysis of human genomic DNA indicated

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only one gene containing the PDI sequence (43). In addition, the isolated ß-subunit of prolyl-4-hydroxylase has PDI activity similar to PDI itself, and even in the intact tetramer form the subunit had 50% of the theoretical activity (43). These data imply that PDI might be involved in the synthesis of collagen. Another cDNA for human p55 protein, defined as thyroid hormone 3,3',5-triiodo-L-thyronine (T3) binding protein, was sequenced and its coding region was 85% homologous to that of rat PDI and 98% homologous to the ß-subunit of human prolyl-4-hydroxylase (44). The p55 protein was also shown to have PDI activity (45). It has been concluded that PDI, the ß-subunit of prolyl-4-hydroxylase and the p55 protein are the products of the same gene (44), and that the PDI/ß-subunit could also play a role in T3 transport pathways in the cell.

In addition, there are some other proteins that are newly described as relatives of PDI. A chicken glycosylation site binding protein, a component of an oligosaccharyl transferase which catalyses the N-linked glycosylation on nascent proteins, shared 90% homology to that of rat PDI in amino acid sequence (46). It is likely that PDI has multiple functions in the modification of newly translated proteins. Sequencing of cDNA clones encoding two abundant lumenal endoplasmic reticulum proteins (ERp), ERp59 and ERp72, revealed that ERp59 is identical to PDI, and that ERp72 showed sequence homology with ERp59/PDI at those regions having copies of the sequence, Cys-Gly-His-Cys, the proposed active site of PDI (47). Another 58 kd protein of the microsomal triglyceride transfer protein complex (MTP) was recently identified as PDI by comparison of the properties of the two proteins with respect to N-terminal sequence, reverse phase HPLC maps,

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immunological reactivities, and enzyme activity (48). It is interesting that all these PDI-like proteins, as well as PDI itself, are located in the lumen of the endoplasmic reticulum where proteins are synthesized and modified. Thus, PDI is thought to be a multifunctional enzyme involved in cotranslational modifications and posttranslational folding of proteins (49). Questions about PDI function still remain to be answered, despite the present state of knowledge. PDI is not a very effective catalyst and rather high enzyme concentrations are needed to observe catalytic protein re-oxidation, in vitro, and the lack of a well defined substrate has hampered the study of the catalytic mechanism for PDI (30). Studies, in vivo, both on the catalytic reaction and on its physiological function are limited. How the multifunctional enzyme is regulated in playing role(s) either alone or associated with other proteins needs further investigation.

Concurrently, a similar enzyme called thiol:protein disulfide oxidoreductase, also named glutathione-insulin transhydrogenase (GIT, EC 1.8.4.2), catalyzing thiol-disulfide exchange reactions in the presence of GSH, was studied by several groups (50-57). This protein was originally isolated as an insulin disulfide reduction activity that yielded a trichloroacetic acid (TCA)-soluble insulin A chain by cleavage of the hormone's disulfide bond with GSH (50, 51). Since then GIT was purified from different species and tissues (50, 52-57). Because GIT is similar to PDI in nearly every respect, for example, in activity, molecular weight, pI, distribution in tissues and subcellular locations, it was argued in the 1970s that PDI and GIT were the same protein. Freedman, et al. co-purified the PDI activity and GIT activity through the stages of the purification from rat liver, and suggested that a

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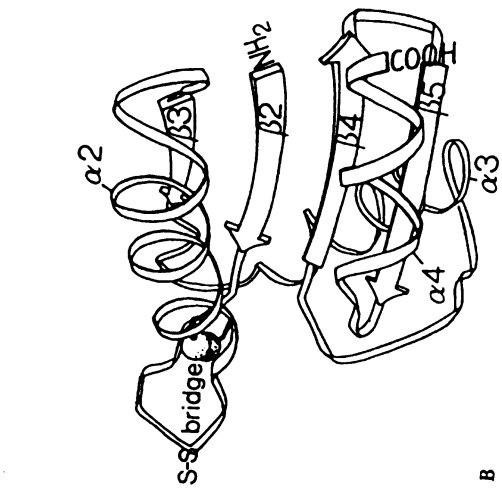
single protein was responsible for both activities (58). The collaborative study of six preparations of GIT and PDI with double immunodiffusion and rocket-line immunoelectrophoresis confirmed the immunological identity of PDI and GIT (59). Today, it is generally accepted that PDI and GIT are identical, and represent two names for the same proteins. Recent observations from our laboratory revealed a further intrinsic function of PDI, namely dehydroascorbate reductase activity (60). Further discussion of this finding is given below.

Thioredoxin

In addition to PDI, three cytosolic low molecular weight proteins, thioredoxin, thioltransferase and glutaredoxin, have the ability to catalyze thiol-disulfide exchange reactions. Among these heat stable enzymes, thioredoxin is the most extensively studied (61, 62).

In 1964, thioredoxin was first described as the hydrogen donor for E. coli ribonucleotide reductase, an enzyme that catalyzes the formation of deoxyribonucleotides from the corresponding ribonucleotide (63). Since then, the ubiquitous protein has been purified from bacteriophage (64), bacteria (63, 65, 67), yeast (68, 69), green algae (70,71), plants (72-74), and animals (75-79). Thioredoxin from all organisms is a single polypeptide with 104-114 amino acids and a molecular weight of approximately 12,000, except that from bacteriophage (T4), which contains 87 amino acids and has no sequence homology and no immunological cross-reactivity with the E. coli enzyme (77, 78). However, X-ray crystallography to 2.8 Å resolution for both proteins showed that they share a similarity in folding and have common three dimensional structures with the active site disulfide bridge in a

Fig. 1. The three dimensional structures of E. coli and T4 thioredoxins. Schematic backbone drawing of three dimensional structures of E. coli (left) and T4 (right) thioredoxins showing their similar folding.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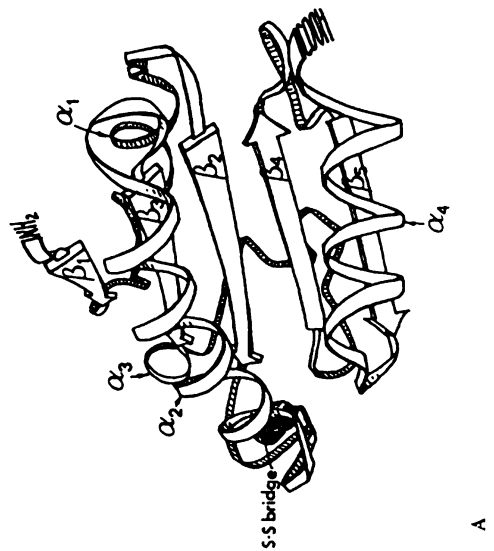
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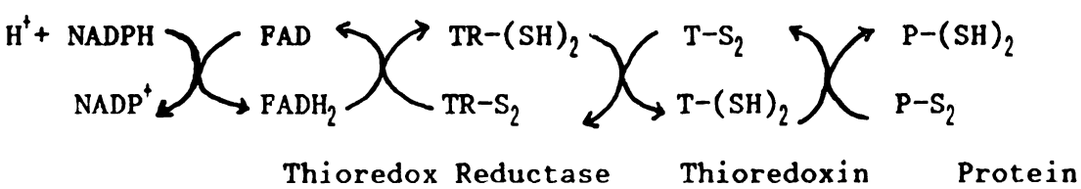
protruding loop (Fig. 1) (82, 83). The primary structures of thioredoxin from different sources were determined either by direct amino acid sequence or deduced from their cDNA sequences with various conserved homologies among them, e.g., all contain the active center sequence, Cys-Gly-Pro-Cys (79, 84-95), implying all the proteins evolved from a common ancestor. The active site sequence, Cys-Val-Tyr-Cys, of T4 thioredoxin is more like that, Cys-Pro-Tyr-Cys, of glutaredoxin. In fact, the two proteins are closely similar in amino acid sequence (96, 97).

The thioredoxin system, including thioredoxin, NADPH, and thioredoxin reductase, a FAD-containing protein with a redox-active disulfide, seems to participate in many diverse biochemical processes as a general protein thiol-disulfide oxidoreductase by transferring electrons from NADPH to its substrate proteins as shown below (61):



For E. coli and mammalian cells, the biochemical function of thioredoxin was originally reported to be a hydrogen donor for ribonucleotide reductase, in vitro, (64, 67). However, additional roles for the enzyme were later described. Pigiet and Schuster found that thioredoxin from E. coli catalyzed the refolding of ribonuclease either from the reduced, denatured form or from the scrambled form, suggesting that thioredoxin may serve as a PDI analogue in E. coli (98). Rat liver

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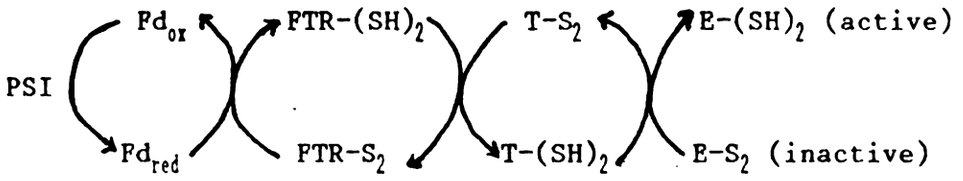
thioredoxin could activate cytosol glucocorticoid receptor activity by maintaining the receptor in reduced form (99). T7 DNA polymerase posesses full activity only in the presence of E. coli thioredoxin, indicating that thioredoxin is an essential subunit of T7 DNA polymerase (100). Thioredoxin also plays significant roles in the assembly of filamentous phages (f1, M13) (101) and in the initiation of protein synthesis (102). The process of reducing insulin disulfides to produce aggregating B chains is currently used to assay thioredoxin activity (103).

Different from E. coli and mammalian cells, plant tissues contain two or more thioredoxin species (61). The best characterized plant thioredoxins are from spinach chloroplasts in which two types of thioredoxin exist, the f- and m- types, classified by their preference for enzyme activation (73). Both types have been purified and sequenced (87, 93), and show little sequence homology (<18%). In green tissues of plants, sunlight provides the energy for photosynthesis and, indirectly, electrons for the regulation of chloroplast enzyme activities (104). Some of the regulatory steps are connected by thioredoxins and the general pathway for thioredoxin-linked regulation is shown below



in which PSI, Fd, FTR, T, and E represent photosystem I, ferredoxin, ferredoxin-thioredoxin reductase, thioredoxin, and target enzyme,

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respectively (105). The well-established examples of such regulation are two photosynthesis enzymes, fructose-1,6-bisphosphatase and $NADP^+$-malate dehydrogenase which are specifically activated by f- and m- type thioredoxins, respectively. The f-type protein also works on $NADP^+$-malate dehydrogenase but with different kinetics (73, 106). Thioredoxin was also shown to be essential for photosynthetic growth in cyanobacterium Anacysis nidulans R_2, in vivo, since deletion of the gene of thioredoxin m is a lethal mutation (107).

Recently, Holmgren's research group demonstrated that PDI from calf liver has intrinsic thioredoxin activity (catalysis of NADPH dependent insulin disulfide reduction) and is a substrate for thioredoxin reductase from calf thymus or rat liver (108). Thus, PDI might be a high molecular weight member of the thioredoxin family. Most observations on the functions of thioredoxin were obtained, in vitro, and the details of how this small enzyme functions in the above biochemical processes were not revealed. Hence, the mechanisms of the reactions and the true functions of thioredoxin in living cells require further studies.

Thioltransferase (Glutaredoxin)

Compared with thioredoxin, thioltransferase has a longer history. In 1955, Racker first discovered an enzyme with an activity that catalyzes the conversion of homocystine to homocysteine in the presence of GSH, GSSG reductase and NADPH, and named it GSH-homocystine transhydrogenase (109). In 1960, a protein with similar activity, but with preference to L-cystine as the substrate, was detected in yeast by Black's group (110), and several years later this protein was highly

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purified and called thiol-disulfide transhydrogenase (111). With the finding of the native unsymmetrical disulfide of coenzyme A and glutathione, CoASSG, Chang and Wilken purified a enzyme, with a molecular weight of 12,000, from bovine kidney, which catalyzed the reduction of CoASSG to CoA and GSH and could not reactivate "scrambled" ribonuclease. They named the protein sulfhydryl-disulfide transhydrogenase (112). All the early reported enzymes catalyzing the GSH dependent reduction of low molecular weight disulfide substrates (reaction 1) were named "transhydrogenases"

$$RSSR + 2GSH \rightleftharpoons 2RSH + GSSG \qquad (1)$$

where RSSR represents widely variable disulfide substrates.

By examination of the mechanism of the above reaction (reaction 1), Mannervik and co-workers, who isolated a "transhydrogenase" from rat liver (113), pointed out that this thiol-disulfide exchange reaction catalyzed by the enzyme followed a thioltransferase mechanism via consecutive ionic displacement reactions

$$RSSR + GSH \rightleftharpoons RSSG + RSH \qquad (2)$$

$$RSSG + GSH \rightleftharpoons GSSG + RSH \qquad (3)$$

rather than the transhydrogenase mechanism, i.e., a one step three substrate reaction (114). The sum of reactions 2 and 3 equals reaction 1. The resulting GSSG can be reduced by GSSG reductase using NADPH as electron donor. They replaced the name "thiol (or GSH)-disulfide transhydrogenase" with "thioltransferase" which has been commonly accepted by most laboratories today.

Characterization of viable E. coli mutants lacking detectable thioredoxin, but still maintaining full ribonucleotide reductase activity resulted in the discovery of glutaredoxin as a GSH-dependent hydrogen donor system including glutaredoxin, GSH, glutathione reductase and NADPH (115). Later, similarities in structure, catalytic activity, size, immunology and other properties between thioltransferase and glutaredoxin indicated that they were identical (see below). TT and GRX have been purified from E. coli (113,116), yeast (111,117), rat liver (113,118), calf thymus (119), bovine liver (120), human placenta (121), pig liver (122), and rabbit bone marrow (123). The molecular weight of thioltransferases or glutaredoxins from animal tissues and yeast is about 11,700, whereas that from E. coli is ca. 10,000; all enzymes are located in the cytosol (124) and have catalytic activity toward various low molecular weight disulfides and protein disulfides in the presence of GSH, GSSG reductase, and NADPH. This reaction is used as a standard assay for thioltransferase thiol-disulfide exchange activity. The primary structures of E. coli (96, 125), calf thymus (126, 127), yeast (117), pig liver (128, 129), and rabbit bone marrow (123) thioltransferases (glutaredoxins) were determined either by direct amino acid sequence (96, 117, 125, 127) or deduced from their nucleotide sequence (125, 129). The three mammalian enzymes contain 105 amino acids (106 for rabbit protein) with two pairs of cysteines and share 85% sequence homology, whereas the yeast and E. coli enzymes contain 106 and 85 amino acids, respectively, with only two cysteine residues and have 32% sequence identity to each other. The sequence similarity between the pig and yeast protein is 51% and that between the calf and E. coli polypeptide is 30%. However, all enzymes have the same active center

with the sequence of Cys-Pro-Tyr(Phe for pig enzyme)-Cys-, located in the N-terminal region, indicating they are probably from the same ancestral gene, especially regarding the mammalian proteins. Immunological studies showed that polyclonal antibodies against pig liver thioltransferase can recognize calf thymus glutaredoxin and calf liver thioltransferase with the same sensitivity, but not E. coli thioredoxin (130). Antibodies against calf thymus glutaredoxin can cross-react with human placenta thioltransferase (131). The experimental data described above strongly support the conclusion that TT and GRX are two names for the same protein.

Functional studies of thioltransferase (glutaredoxin) were also performed in several laboratories, mainly in Mannervik's group. Mannervik, et al. (8) reported that the synthesized mixed disulfide between lysozyme and GSH (or Cysteine), or trypsin and GSH were reduced in the presence of thioltransferase, but not GSSG reductase and NADPH. This group further reported that thioltransferase could reactivate pyruvate kinase which had been inactivated previously by treatment with GSSG (132), and fully protect the enzyme from oxidative inactivation in air in the presence of GSH, GSSG reductase, and NADPH (133). The thioltransferase and thioredoxin systems were compared with regard to reduction of various disulfide substrates. Thioltransferase was more efficient in reducing small molecular weight disulfide, whereas thioredoxin had a higher affinity for protein substrates (134). A Japanese research team observed that thioltransferase could restore the activity of papain, which was previously inactivated by oxidation, in the presence of GSH, in vitro (135). Goswami et al. (24) described a cytosolic protein (glutaredoxin) having GSH-disulfide "transhydrogenase"

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activity that could activate microsomal iodothyronine deiodinase, a microsomal enzyme that catalyzes the *in vitro* conversion of thyroxine (T4) to triiodothyronine (T3) in the presence of GSH. Recently, it was reported that the thioltransferase and thioredoxin systems could protect the essential thiol groups of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis (136). A novel function was recently assigned to mammalian thioltransferase as well as PDI in this laboratory, namely GSH dependent dehydroascorbate reductase activity (60).

Glutaredoxin (thioltransferase) is a general GSH dependent thiol disulfide oxidoreductase capable of interacting with ribonucleotide reductase (RRase). The affinity of *E. coli* glutaredoxin to RRase is ten times higher than that of thioredoxin, whereas the concentration of thioredoxin in these cells is ten times higher than that of glutaredoxin (62). However, neither thioredoxin nor glutaredoxin is essential for RRase activity because an *E. coli* mutant, lacking both enzymes, was viable, but required supplemental cysteine for growth on a minimal medium, indicating the presence of a third unknown electron donor for RRase (62). Mammalian glutaredoxins (thioltransferases) from different sources display different behavior as electron donors for RRase. The calf thymus enzyme is active in the homologous RRase system in the presence of GSH, GSSG reductase, and NADPH (137), but neither the rabbit bone marrow enzyme nor pig liver enzyme served as electron donor for rabbit bone marrow RRase, and rabbit bone marrow thioredoxin also did not play such a role (123). These results raise the question, what is the electron donor for RR in rabbit bone marrow and pig liver? What roles do the two proteins play, *in vivo*? Generally, thioltransferase

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may play a role in the regulation of the biological activity of the thiol status of proteins or enzymes by reversible modification, but there is a shortage of meaningful *in vivo* data. Now, it is generally speculated that the membrane associated PDI functions mainly on protein thiol-disulfide rearrangement, whereas both thioredoxin and thioltransferase regulate some preferred protein (enzyme) thiol status using different electron donor systems. For low molecular weight disulfides thioltransferase mediated reactions are the most likely (131). It is of interest that no thioltransferase (glutaredoxin) has been reported in plants so far, although a DHA reductase, quite different from thioltransferase in properties, has been described (138).

The three established thiol-disulfide oxidoreductases contain an active site with the sequence of CysxxCys, i.e., the two cysteines are separated by two amino acids in a 14 atom loop. As stated above, there are two sequences, Cys-Gly-His-Cys, in PDI although it is not established whether one or both represent the active site or active sites, respectively. The active sites of thioredoxin and thioltransferase were identified as Cys-Gly-Pro-Cys (137) and Cys-Pro-Tyr(Phe for pig enzyme)-Cys, respectively. Two more cysteines, separated by three amino acids, are found near the C-terminus of thioredoxin and thioltransferase in mammalian tissues. The function of the second pair of cysteines is not clear.

The active site and reaction mechanism of the two low molecular weight enzymes have been studied in recent years. Holmgren's group (139) identified Cys^{32} as the active site of *E. coli* thioredoxin by kinetic studying of the alkylation reaction between the reduced protein and iodoacetic acid (IAA) or iodoacetamide (IAM) and by the radioactive

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labeling of the active site. They found that the sulfhydryl group of Cys^{32} has a low pK_a of 6.7 and only Cys^{32} was labeled by [^{14}C]IAA at a pH of 8.3. From these studies, a mechanism for thioredoxin as a protein disulfide reductase was proposed (139). Recently, several active site mutant T4 (140) and E. coli thioredoxins (141) were constructed and used to investigate their influences on enzyme activity. The active site, Cys^{14} Val Tyr Cys^{17} of T4 thioredoxin was modified to construct the mutants, Cys-Gly-Pro-Cys (same as E. coli), Cys-Val-Pro-Cys, and Cys-Gly-Tyr-Cys. The three mutants had similar reaction rate with T4 RRase. The mutants without Tyr at position 16 were more like E. coli thioredoxin with respect to K_m for E. coli RRase, while those without a Val at position 15 had quite similar properties to those of the wild T4 enzyme (140). Gleason, et al. constructed two mutants, Cys-Gly-Arg-Pro-Cys and Cys-Ala-Cys at the active site, i.e., Cys-Gly-Pro-Cys, of E. coli thioredoxin by altering the size, and demonstrated that the mutant with the larger size lost 85% activity, while that with the smaller size had no activity (141). The mutation studies suggested that the distance rather than the amino acids between the active site cysteines was more essential. The extra two cysteines in mammalian enzymes are separated by three amino acids and are presumably not the active site.

Similar studies on thioltransferase have been carried out. Pig liver thioltransferase has been purified to homogeneity with a molecular weight of 11,700 (122). Its primary structure was determined by conventional amino acid sequence analysis to contain 105 amino acids (128). The kinetic study of the alkylation reaction between equal amount of thioltransferase and IAM showed that the pK_a for the Cys^{22} sulfhydryl group is extremely low with a value of 3.8 (142). [^{14}C]IAA

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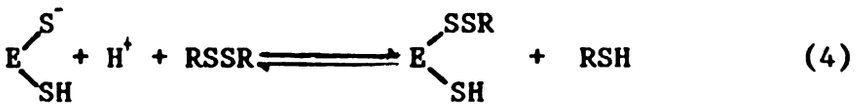
labelling of the pig enzyme showed that only Cys^{22} was labeled and suggested that the active site for this enzyme is located at Cys^{22} (143). Based on the observation that the enzyme, in its oxidized form (intramolecular disulfide form) caused by pre-incubation with hydroxyethyl disulfide (HED) or cystine, could be fully protected from inhibition by IAA, a reaction mechanism for TT was proposed (143) as follows:



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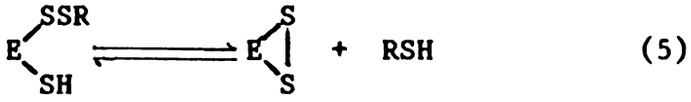

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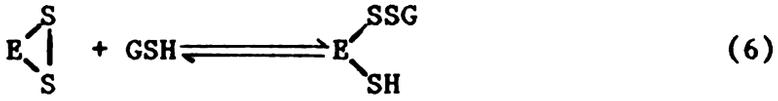

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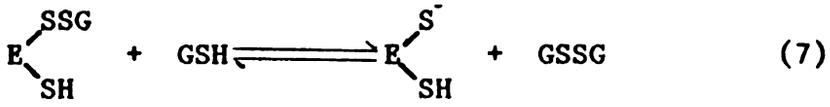

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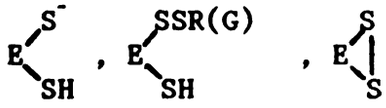
where  , RSSR, represent thioltransferase in the reduced form, oxidized form with mixed disulfide, oxidized form with intramolecular disulfide, and disulfide substrate, respectively.

However, the proposed active site and reaction mechanism of thioltransferase were based on indirect evidence. In addition, the reason for the extremely low pK_a of Cys^{22} and the roles of certain amino acids at the active center were not clear. Hence, direct evidence and further investigations were required to establish the active site and

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the mechanism of the enzyme. Site directed mutations of amino acids at the proposed active site is the favored direct way to examine this speculation. At the time this dissertation was started in 1987, no molecular cloning work of thioltransferase was reported except that in E. coli (125). The bacterial glutaredoxin gene, grx, contains 1147 base pairs and its open reading frame sequence of 255 base pairs gave a deduced amino acid sequence identical to the directly determined one (96). The goals of this dissertation were to clone the mammalian thioltransferase gene (cDNA), establish an efficient expression system, and investigate the active site and catalytic mechanism of thioltransferase.

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CHAPTER II

CLONING AND SEQUENCING THE cDNA ENCODING PIG LIVER THIOLTRANSFERASE

SUMMARY

The amino acid sequence of pig liver thioltransferase has been published. We report here the first successful cloning and sequencing of full length cDNA of the pig liver thioltransferase gene. The cDNA for thioltransferase was obtained by screening a commercial (Clonetech) pig liver cDNA library in λ-gt11 using polyclonal antibodies raised in rabbits against pig liver thioltransferase. Two positive clones were identified in 3.5×10^5 recombinants. For verification, we successfully hybridized three oligonucleotide probes, synthesized according to three different regions of the pig liver thioltransferase amino acid sequence, to the two clones. In addition, the size of the thioltransferase-ß-galactosidase fusion protein, produced by the positive clone, was consistent with the length of the cDNA. The thioltransferase cDNA was subcloned into the EcoR1 site of M13mp18 and sequenced by the dideoxy chain termination method using ^{35}S-labeled nucleotide. The amino acid sequence deduced from the cDNA sequence is in exact agreement with the previously reported primary amino acid sequence, except that the N-terminus should be N-acetylalanine followed by glutamine rather than the reverse as originally interpreted by conventional mass spectrometry fast atom bombardment analysis of the tryptic peptide corresponding to the first 8 amino acid residues.

INTRODUCTION

Thioltransferase is an enzyme that reduces a variety of disulfides, including protein disulfides, in the presence of reduced glutathione (1-3). The enzyme has been purified and partially characterized from rat (3), yeast (4), calf (5, 6), pig (7) and human (8) sources. It was suggested that thioltransferase could be a general protein disulfide reductase with the function of keeping various cellular proteins in their reduced forms (2, 9). Thus thioltransferase may affect some enzyme activities, such as pyruvate kinase (10) or papain (11) under physiological conditions.

Thioltransferase, purified to homogeneity from pig liver (7), consists of 105 amino acids and its primary structure was determined by conventional amino acid sequence analysis (12). The active site of the enzyme was identified as a 14 atom loop at positions, $-Cys^{22}-Pro^{23}-Phe^{24}-Cys^{25}-$. The pK_a value of Cys^{22} sulfhydryl group was found to be 3.8 (13, 14), which may explain its increased S-nucleophilicity in the 2-step ionic displacement reactions characteristic of dithiol-disulfide exchange reactions (15). In addition to studies that report similar catalytic properties between pig liver thioltransferase and calf thymus glutaredoxin (7, 16, 17), immunological studies and amino acid sequence comparison of the two enzymes show that these two proteins are essentially identical (18). Previous work on thioltransferase has focused on its purification, substrate specificity, kinetics, primary structure and function (1-8,10-19).

Recently, we have reported preliminary evidence for cloning and sequencing the cDNA of the pig liver thioltransferase gene (20). In this article we extend this study and describe in detail the first successful

cloning and sequencing of the full length cDNA of the pig liver thioltransferase gene. The amino acid sequence deduced from the cDNA sequence is compared to the previously published amino acid sequence (7).

MATERIALS AND METHODS

Materials-- A pig liver cDNA library in λ-gt11 (Lot no. 1022), λ-gt11 chicken ovalbumin clone and E. coli Y1090 were purchased from Clontech Laboratories Inc.. Rabbit antiserum against pig liver thioltransferase was prepared as previously described (18). Rabbit anti-E.Coli ß-galactosidase antibody, goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate, AP color development reagent-BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium), nitrocellulose membrane, Tween-20, protein molecular weight markers and calf alkaline phosphatase are from Bio-Rad. Nitrocellulose filters were from Schleicher & Schuell. Pig liver thioltransferase was purified as described (7). Restriction enzyme EcoR1, IPTG (isopropyl ß-D-thiolgalactopyranoside) and X-gal were from Boehringer Mannheim Biochemicals(BMB). T4 DNA ligase and EcoR1 digested M13mp18 RF DNA were from New England Biolabs. Other standard reagents used in this work were from Sigma, Bio-Rad, Difco Laboratories, BMB and BRL.

Antibodies Screening of Thioltransferase cDNA Clones-- Approximately 3.5 X 10^5 plaque-forming units (pfu) from the pig liver cDNA library in λ-gt11 were plated on seven 150 mm Petri dishes and screened by using rabbit polyclonal antibodies against pig liver thioltransferase (18) essentially as described (21). Briefly, after the plaques growth, each of the seven plates was overlaid with two IPTG treated nitrocellulose filters separately. The bound proteins were reacted with the 1:50 diluted anti-thioltransferase antibody for one hour and then incubated in 1:7500 diluted alkaline phosphatase-conjugated goat anti-rabbit IgG for one more hour. Both antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20 (TBST) solution. Finally, the filters were washed in TBST

(10 min X 3) followed by visualization with color development substrate (NBT + BCIP) solution (22). Three positive signals were found, and their corresponding plaques in the original plates were replated and rescreened at a low plaque density (200 pfu/plate) using the same strategy as the primary screening. Only two of them showed multiple positive clones.

Oligonucleotide Probes Hybridization-- For verification of the two positive clones, three oligonucleotide probes were designed and synthesized (23) according to three different regions of the known amino acid sequence of pig liver thioltransferase. Each of the probes is a mixture of oligonucleotides containing all the nucleotide sequences which are complementary to all the possible codon combinations for its corresponding amino acid sequence (Table II). Probe 1 and probe 3 are 20-mers corresponding to the amino acid sequences of -Lys^{19}-Pro-Thr-Cys-Pro-Phe-Cys^{25}- and -Phe^{73}-Ile-Gly-Lys-Glu-Cys-Ile^{79}- respectively, while probe 2 is a 17-mer corresponding to the amino acid sequence of -Asn^{54}-Glu-Ile-Gln-Asp-Tyr^{59}-. A single well isolated positive clone was picked from each of the two rescreened low plaque density plates and hybridized with the three probes based on a protocol provided by the BRL, Inc. (24). Briefly, the two single positive clones were replated separately, grown overnight and were transferred to nitrocellulose filters. The filters were then prehybridized with Salmon sperm DNA (from Sigma), hybridized with the ^{32}P-labeled oligonucleotide probes, washed with 6 X SSC (1 x SSC, 150 mM sodium chloride and 15 mM sodium citrate) with 0.05% sodium pyrophosphate for 1 hour at 37^{0}C and for 10 min at 50^{0}C, and exposed to X-ray film (Kodak XAR-5) with an intensitifying screen at -70^{0}C for two days. Both of the positive clones hybridized to all three probes (data not shown).

Table II Oligonucleotide Probes for Hybridization of cDNA for Pig Liver Thioltransferase

	19	20	21	22	23	24	25
Amino Acid Seq.	Lys	Pro	Thr	Cys	Pro	Phe	Cys
Possible Codons	5'AA$^{A}_{G}$	CCN	ACN	UG$^{U}_{C}$	CCN	UU$^{U}_{C}$	UG$^{U}_{C}$ 3'
Probe 1	3'TT$^{T}_{C}$	GGN	TGN	AC$^{A}_{G}$	GGN	AA$^{A}_{G}$	AC 5'

	54	55	56	57	58	59
Amino Acid Seq.	Asn	Glu	Ile	Gln	Asp	Tyr
Possible Codons	5'AA$^{U}_{C}$	GA$^{A}_{G}$	AU$^{U}_{C}_{A}$	CA$^{A}_{G}$	GA$^{U}_{C}$	UA$^{U}_{C}$ 3'
Probe 2	3'TT$^{A}_{G}$	CT$^{T}_{C}$	TA$^{A}_{G}_{T}$	GT$^{T}_{C}$	CT$^{A}_{G}$	AT 5'

	73	74	75	76	77	78	79
Amino Acid Seq.	Phe	Ile	Gly	Lys	Glu	Cys	Ile
Possible Codons	5'UU$^{U}_{C}$	AU$^{U}_{C}_{A}$	GGN	AA$^{A}_{G}$	GA$^{A}_{G}$	UG$^{U}_{C}$	AU$^{U}_{C}_{A}$ 3'
Probe 3	3'AA$^{A}_{G}$	TA$^{A}_{G}_{T}$	CCN	TT$^{T}_{C}$	CT$^{T}_{C}$	AC$^{A}_{G}$	TA 5'

Note: N represents A, G, C and U (T).

Western Blot Analysis of the Fusion Protein-- For further confirmation, the thioltransferase-ß-galactosidase fusion protein made by one of the positive clones proven by hybridization was analyzed using the immunoblotting technique modified according to Huynh et al. (25). The λ-gt11 recombinant lysogen was induced as follows. This clone was amplified to approximately 10^{10} pfu/ml (26). The amplified recombinant λ-gt11 solution (5 ul) was dropped onto an E. coli Y1090 lawn in a LB plate (pH 7.5) and incubated at 30°C overnight. E. coli Y1090 from the center of the large "plaque" was streaked out onto a new LB plate (pH 7.5) and incubated at 30°C overnight. Ten well-isolated colonies from the plate were spotted in parallel onto two new LB plates (pH 7.5). One of the plates was incubated at 30°C and the other at 42°C for 5-6 hours. Colonies which grew at 30°C but not at 42°C were defined as induced lysogens. One of the E. coli Y1090 recombinant lysogen strains was picked up and incubated in LB medium (pH 7.5) at 30°C until $O.D._{600}$ = 0.5. At this point IPTG and PMSF (Phenylmethylsulfonyl Fluoride) was added into the culture to 10 mM and 1 mM final concentration, respectively, and the culture was continuously incubated at 37°C until the culture was clear (ca. one hour). The proteins were collected by 70% ammonium sulfate precipitation and resolved in TEP (100 mM Tris-HCl pH7.5, 10 mM EDTA and 1 mM PMSF) buffer. The proteins were separated on 15% SDS-polyacrylamide gels (27). One part of the gel was stained with Coomassie brilliant blue and the other two parts were transferred to nitrocellulose membranes (28) at a constant current of 160 mA for 2 hours in transfer buffer (12.5 mM tris-HCl, 96 mM glycine, 0.02% SDS and 20% methanol, pH 8.3). One of the two segments was treated with the anti-thioltransferase serum, while the other was treated with anti-ß-galactosidase followed by the AP conjugated secondary antibody visualizing

reaction. The thioltransferase purified from pig liver (7) and E. coli ß-galactosidase (116.3 kDa) in Bio-Rad High Molecular Weight Standard were used as positive controls, and Y1090 not infected by the λ-gt11 was used as a negative control.

DNA sequence Analysis-- The identified recombinant λ-gt11 that contained the pig liver thioltransferase cDNA was amplified as described (26), and its DNA was purified using an ammonium sulfate precipitation method (29). The purified recombinant λ-gt11 DNA was digested with EcoR1 (2 Unit/ug DNA) at 37°C for 2 hours and electrophoresed preparatively on a 5 % polyacrylamide gel followed by ethidium bromide staining. The lowest band (about 600 base pairs) was cut out and the 600 base pairs DNA fragment (the thioltransferase cDNA) was released from the gel in elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.2% SDS) (30), extracted by phenol/chloroform and precipitated with absolute ethanol (31). The cDNA was subcloned into M13mp18 by ligation of the cDNA and EcoR1 digested M13mp18 replicative form (RF) DNA with T4-ligase. The recombinant M13mp18 RF DNA was introduced into E.coli JM101 via transformation of the competent cells. The amplified recombinant M13mp18 RF DNA and single stranded DNA were prepared essentially as described (32). The RF DNA was digested with EcoR1 (2 Units/ug DNA) again and analyzed on 5 % polyacrylamide gel electrophoresis to confirm that the thioltransferase cDNA was inserted. In addition, the M13mp18-thioltransferase recombinant RF DNA was digested with Pst1 and the largest enzymatically generated DNA fragment (M13mp18 + 3' region of the cDNA) was isolated by polyacrylamide gel electrophoresis. E. coli JM101 was transformed with the religated M13mp18 containing the partial thioltransferase cDNA. The whole length cDNA and the 3' region of the

cDNA were sequenced by Sanger's method (33) under the conditions recommended in BRL's KiloBase Sequencing System (34) using ^{35}S-dATP (Amersham) as the labeled nucleotide.

RESULTS AND DISCUSSION

Antibody Screening of Thioltransferase cDNA-- A commercial (Clontech Laboratory Inc.) pig liver cDNA library in λ-gt11 was screened using rabbit polyclonal antibodies against pig liver thioltransferase and three positive clones were observed in 3.5 X 10^5 recombinants. Separate rescreening of the three clones with the same antibody in a low pfu density (about 200 plaques/plate) revealed that only two of them gave a number of positive signals. During the primary screening, we noticed that the color of the false positive signal was weaker than that of the other two. This phenomenon might be caused by the nonspecific binding of the antibodies and bacterial proteins, though the rabbit antiserum was pre-treated with E. coli extract to decrease the background. At the same time, a control experiment using pure λ-gt11 clones containing chicken ovalbumin DNA sequences (Clontech Laboratories Inc.) was performed. We treated these clones using antibody against chicken ovalbumin and the anti-thioltransferase antibody as the primary antibody separately, only the former showed positive signals, whereas the latter were negative. Accordingly, the antibody against pig liver thioltransferase (18) used in the present study has a reasonably high specific affinity and the two positive clones obtained by the antibody-screening appeared reliable.

Oligonucleotide-Probe Hybridizations-- To confirm the positive clones screened by antibody, three oligonucleotide probes (Table II) which were labeled with ^{32}P at the 5'-end were used to hybridize to the two positive clones. Both of the clones gave positive hybridization reactions with the three probes (data not shown). Because each of the probes is an oligonucleotide mixture which contains the nucleotide sequences complementary to all the possible codon combinations for its corresponding

amino acid sequence, the probes were expected to hybridize to the two clones if they contained the pig liver thioltransferase cDNA. In addition, the three peptide sequences selected for the probes were located near the N-terminus, at the middle and near the C-terminus of pig liver thioltransferase, respectively. After successfully hybridizing the three probes to the clones separately, this sequence distribution helped to establish that the cDNA inserted into the λ-gt11 clone was likely a complete rather than partial sequence. The hybridization experiment showed that the two antibody-positive clones were hybridized by all three oligonucleotide probes, whereas the pure chicken ovalbumin cDNA was not hybridized by any of the probes. These results verified that two λ-gt11 clones having the full-length thioltransferase cDNA were obtained.

Western-blotting Analysis the Fusion protein-- One of the positive clones was further analyzed by western blotting of the thioltransferase-ß-galactosidase fusion protein produced by this clone. The pig liver thioltransferase cDNA was inserted into the λ-gt11 lacZ gene, coding for ß-galactosidase, at the EcoR1 site (Clontech Product Profile). When the recombinant λ-gt11 infects its host cell, the thioltransferase-ß-galactosidase fusion protein, the product of the recombinant lacZ gene, will be made (35). The expression vector λ-gt11 makes a temperature-sensitive repressor (cI857) which is inactive at 42°C and has an amber mutation (S100) which only lyses the hosts containing the amber suppressor supF (25). On the other hand, E. coli Y1090 has a lac repressor which inhibits lacZ gene expression until derepressed by IPTG, a deficient lon protease which decreases the degradation of the recombinant fusion protein, and supF which suppresses the phage mutation (S100) (21). Thus, λ-gt11 will not lyse Y1090 at the incubation temperature of 30°C because

of its cI857 mutation, and lacZ won't be expressed until the addition of IPTG. Taking advantage of these characteristics, we successfully induced the Y1090 lysogen strains by infecting the cells with the recombinant λ-gt11 and obtained sufficient thioltransferase-ß-galactosidase fusion protein for western blotting analysis (Fig. 2). The fusion protein produced by the recombinant λ-gt11 with the inserted thioltransferase cDNA will have a higher molecular weight than that of E. coli ß-galactosidase alone and react with both the antibody against pig liver thioltransferase and the antibody against E.coli ß-galactosidase. Indeed, immunoblotting analysis of the fusion protein gave these results (Fig. 2). Bands in Panel A were visualized by Coomassie blue staining, while the bands in Panel B and C were visualized by treating with the anti-thioltransferase and the anti-ß-galactosidase (Bio Rad), respectively. It can be seen that the fusion protein (Lane 2 in Panel A, B and C) has a higher molecular weight than E. coli ß-galactosidase (M_r = 116.3 kDa) (Lane 1 in Panel A and C) and reacts with the anti-thioltransferase (Lane 2 in Panel B) and the anti-ß-galactosidase (Lane 2 in Panel C). In contrast, the Y1090 control (Lane 3 in Panel A, B and C) had no bands corresponding to the fusion protein. Therefore, the western-blotting analysis of the fusion protein presents further strong evidence that we had obtained the full-length pig liver thioltransferase cDNA in λ-gt11.

Nucleotide Sequence Analysis-- The positive recombinant λ-gt11 clone containing pig liver thioltransferase cDNA, as judged by antibody-screening, oligonucleotide hybridization and western-blotting analysis, was amplified, and the recombinant λ-gt11 DNA was purified (see Experimental Procedures). The purified DNA was digested with EcoR1 and the enzymatic DNA fragments were separated on a 5 % polyacrylamide gel

Fig. 2 Western blotting analysis of the fusion protein. The preparation of the fusion protein and the performance of western blotting were described in the Experimental Procedures. Panels A, B and C were visualized with Coomassie Brilliant Blue staining, polyclonal antibodies against thioltransferase and antibody against ß-galactosidase respectively. In Panels A, B and C: lane 1, Bio-Rad high molecular weight protein standard, the second band from the top also serves as a ß-galactosidase positive control; lane 2, E. coli Y1090 lysogen extract (fusion protein); lane 3, E. coli Y1090 extract (as negative control); lane 4, pig liver thioltransferase. Lane 5 in Panel A, Bio-Rad low molecular weight protein standard. FP, ß-Gal and T.T. indicate the positions of fusion protein, ß-galactosidase and thioltransferase, respectively.

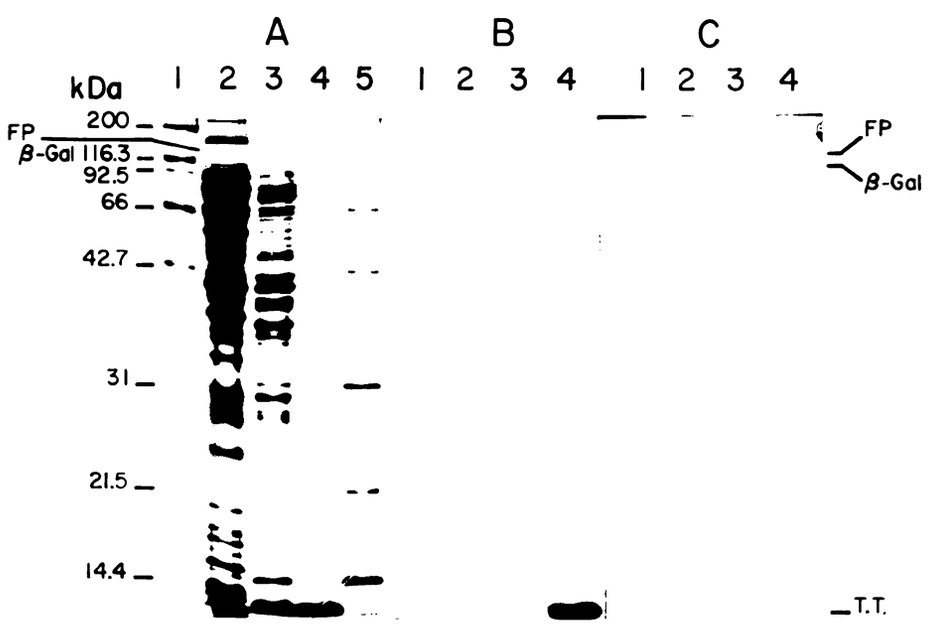
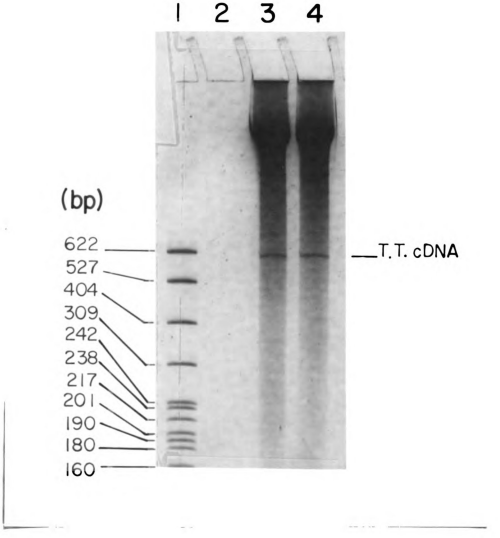

A
B
C
kDa
1 2 3 4 5 1 2 3 4 1 2 3 4
FP
β-Gal 116.3
200
92.5
66
42.7
31
21.5
14.4
FP
β-Gal
T.T.

Fig. 3 Polyacrylamide gel electrophoresis isolation of pig liver thioltransferase cDNA. The λ-gt11/thioltransferase recombinant DNA was digested with EcoR1 and the enzymatic fragments were separated in 5 % polyacrylamide gel for 1 hour at a constant current of 30 mA. Lane 1, DNA molecular size marker (pBR322/HpaII), Lane 2, empty; Lane 3 and 4, λ-gt11/thioltransferase recombinant DNA (50 ug/lane). T.T.cDNA indicates the position of thioltransferase cDNA.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(bp)
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527
404
309
242
238
217
201
190
180
160
T. T. cDNA

(Fig 3). The length of the cDNA was expected to be less than 1000 base pairs since pig liver thioltransferase only has 105 amino acids (12) whereas λ-gt11 consists of 43.7 Kb and has only one EcoR1 site (25). The lowest band (Lane 3 and 4 in Fig 3) had approximately 630 base pairs and was therefore a likely candidate for the thioltransferase cDNA. This fragment was subcloned into a M13mp18 at the EcoRI site, and the insertion was confirmed by EcoRI digestion of the recombinant M13mp18 DNA and analysis on a 5% polyacrylamide gel (data not shown). The corresponding single stranded M13mp18 DNAs, as the template, were sequenced by using the dideoxy chain termination method (33, 34), and the sequencing strategy was shown in Fig. 4. The whole nucleotide sequence of the cDNA for pig liver thioltransferase is shown in Fig. 5. The EcoR1 recognition sequence, -GAATTC- (5'-3'), at both the 5' and 3' ends, was found, and the whole length of the cDNA was 635 base pairs (including the two EcoR1 sites). The open reading frame has 321 base pairs starting with the initiation codon, ATG, and ending with the termination codon, TAA. The open reading region is flanked by two untranslated regions, an upstream sequence (23 base pairs) and a downstream sequence (291 base pairs). The downstream noncoding region has a long poly(A) tail of 57 A residues. Instead of the common poly(A) signal, AATAAA, we found the sequence, ATTAAA, 21 base pair upstream from the poly(A) tail. This indicates that a single base difference in the sequence, AATAAA, still provides the appropriate signal for poly(A) addition (36). The amino acid sequence of thioltransferase deduced from the open reading frame is the same as the previously reported amino acid sequence of pig liver thioltransferase (12) except at the N-terminus. The previously reported N-terminal amino acid sequence was Ac-Gln-Ala-Ala- (Fig. 6) which was

Fig. 4 Pig liver thioltransferase cDNA sequencing strategy. The dark bar represents the coding region of the cDNA and the straight line represents the 5'- and 3'- noncoding regions. The vertical arrows indicate EcoR1 or Pst1 sites used in the cDNA subcloning and sequencing. The horizontal arrows indicate the direction and extent of sequencing.

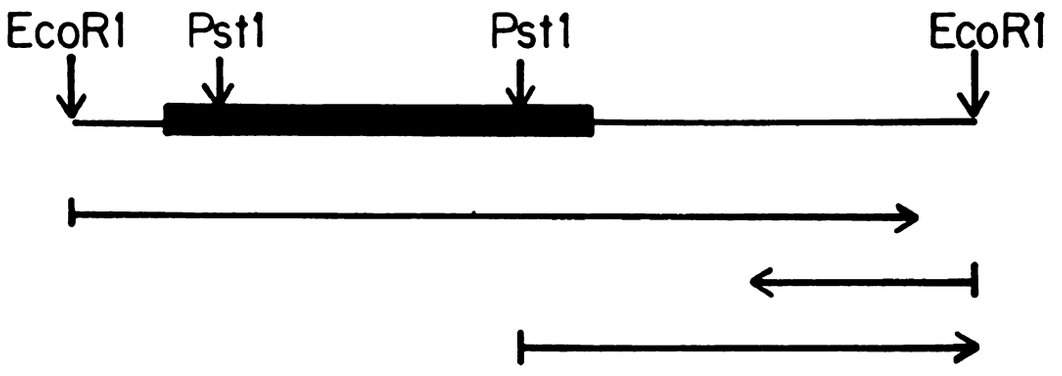

EcoR1
Pst1
Pst1
EcoR1

Fig. 5 Nucleotide sequence of pig liver thioltransferase and its deduced amino acid sequence. The nucleotides are numbered from 5' to 3' at the right of each row, and the amino acids are numbered in brackets from N to C termini. The poly(A) tail signal sequence, ATTAAA, is boxed. EcoR1 sites are underlined; nucleotides in parentheses are not included in the 635 nucleotides of the cDNA.

(G)AATTCCGGCAGCCGCCTGTCAGC 23

ATG GCT CAA GCA TTT GTG AAC AGC AAA ATC CAG CCT GGG AAG GTG 68
Met Ala Gln Ala Phe Val Asn Ser Lys Ile Gln Pro Gly Lys Val [15]

GTA GTT TTC ATC AAG CCC ACC TGC CCC TTC TGC AGA AAG ACA CAG 113
Val Val Phe Ile Lys Pro Thr Cys Pro Phe Cys Arg Lys Thr Gln [30]

GAG CTC CTC AGC CAA TTG CCC TTC AAA GAA GGG CTT CTG GAA TTT 158
Glu Leu Leu Ser Gln Leu Pro Phe Lys Glu Gly Leu Leu Glu Phe [45]

GTC GAT ATT ACA GCC ACC AGT GAC ACC AAC GAG ATT CAA GAT TAT 203
Val Asp Ile Thr Ala Thr Ser Asp Thr Asn Glu Ile Gln Asp Tyr [60]

CTG CAA CAG CTC ACA GGA GCC AGA ACG GTA CCT CGG GTC TTT ATC 248
Leu Gln Gln Leu Thr Gly Ala Arg Thr Val Pro Arg Val Phe Ile [75]

GGT AAA GAG TGT ATA GGT GGA TGC ACT GAT CTA GAA AGT ATG CAC 293
Gly Lys Glu Cys Ile Gly Gly Cys Thr Asp Leu Glu Ser Met His [90]

AAG AGA GGG GAG CTC TTG ACC CGC CTG CAG CAA ATT GGA GCT CTG 338
Lys Arg Gly Glu Leu Leu Thr Arg Leu Gln Gln Ile Gly Ala Leu [105]

AAA TAA TTA CAGCGAGGCAGACCCAAGCTGATAGCTCCCTGTAGAGCTGGATGGCA 394
Lys *

GTGCAGATAATGACAGCGCTTCCTGGTGGATGGATGCCGGGCTACCTTCACTCAGCTGC 453

AACTACTGTTTACTTAAAAATTCTGAAATGTGTTAACCCAAATAATTGGGGGGAGTGGG 512

TTTTGGGGGACAAAACAGATTTTTCTTCTGACTCTGT ATTAAA AGTGGAATCAATCT 569

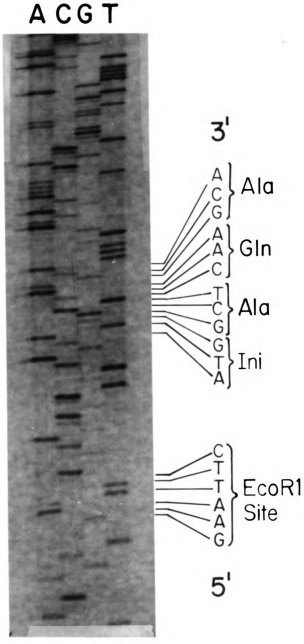
TGCCCC(A)$_{57}$CGG(AATTC) 635

interpreted by conventional fast atom bombardment mass spectrometry (FAB-MS) examination of the T1 peptide, a tryptic octapeptide (12). This initial assignment was based on the observation of peaks at m/z 171 and at m/z 242 in the FAB-MS spectrum which were interpreted as representing B_1 and B_2 fragment ions, respectively. However, it was subsequently determined that the peak at m/z 171 represented extraneous material in the sample and did not represent a B_1 ion from the octapeptide. In contrast, the N-terminal amino acid sequence deduced from the cDNA sequence, which was clearly shown in the sequencing gel film (Fig. 7), was Met-Ala-Gln. The final N-terminal sequence of Ac-Ala-Gln appearing in pig liver thioltransferase results from the post-translational cleavage of the Met and modification of the Ala by acetylation. Glutaredoxin from calf thymus (17, 37) and rabbit bone marrow (38) has also been sequenced and found to have the same N-terminus, N-acetylalanylglutamine. Thus, thioltransferase and glutaredoxin appear to be two names for the same protein based on similarity of amino acid sequence, immunochemical cross-reactivity, and other enzyme properties. Although the glutaredoxin gene (*grx*) of *E. coli* has been cloned and characterized in M13mp9 (39), the present study represents the first successful cloning and sequencing of the cDNA for a mammalian thioltransferase (glutaredoxin), and now provides a powerful tool to further study the structural and functional relationships of the pig liver enzyme.

Fig. 6 Comparison of the N-terminal amino acid sequences of pig liver thioltransferase, calf thymus and rabbit bone marrow glutaredoxin. The nonmatched amino acid (Glu) is boxed. T.T., thioltransferase; Grx, glutaredoxin. * Sequence obtained by amino acid sequencing (12); @ Sequence deduced from the cDNA sequence (this study).

Pig T.T.*	(Ac-)Gln	Ala	Ala	Phe	Val	Asn	Ser	Lys
Pig T.T.@	(Met)Ala	Gln	Ala	Phe	Val	Asn	Ser	Lys
Calf Grx	(Ac-)Ala	Gln	Ala	Phe	Val	Asn	Ser	Lys
Rabbit Grx	(Ac-)Ala	Gln	Glu	Phe	Val	Asn	Ser	Lys

Fig. 7 Autoradiography of the 5'-nucleotide sequence of the cDNA for pig liver thioltransferase. From the bottom to the top is the direction of 5' to 3'. The 5'-EcoR1 recognition sequence and the codons for the N-terminal amino acids of thioltransferase are indicated on the right.


A C G T
3'
A
C
G
Ala
A
A
C
Gln
T
C
G
Ala
G
T
A
Ini
C
T
T
A
A
G
EcoR1
Site
5'

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CHAPTER III

HIGH-LEVEL EXPRESSION OF PIG LIVER THIOLTRANSFERASE (GLUTAREDOXIN) IN *ESCHERICHIA COLI*

SUMMARY

We report the first high-level expression of a mammalian thioltransferase (glutaredoxin) in Escherichia coli. A NcoI site (CCATGG) was introduced into a cDNA encoding pig liver thioltransferase (glutaredoxin) by site-directed mutagenesis, in which the first G of the original sequence, GCATGG, was replaced by a C. The altered cDNA was cloned into an expression vector, plasmid pKK233-2, between the unique NcoI and HindIII sites and expressed in E. coli JM105 at a high-level (8% of total soluble protein) after 6 h of isopropyl-β-D-thiogalactopyranoside induction. The soluble and unfused product was measured by the thioltransferase thiol-disulfide exchange assay and immunoblotting analysis. The recombinant enzyme was purified to a single band as judged by SDS-polyacrylamide gel electrophoresis and isoelectric focusing. The amino acid composition of the expressed enzyme agreed with that of the known sequence of pig liver thioltransferase (glutaredoxin). N-terminal sequence analysis revealed that unlike the native pig liver protein which is N-acetylated, the unfused recombinant enzyme was unblocked at the N-terminus (alanine). Various kinetic properties of the recombinant enzyme with regard to the exchange reaction were identical with those of the native enzyme.

INTRODUCTION

Thioltransferase, also known as glutaredoxin, is a cytosolic enzyme that catalyzes the reduction of disulfides of various molecular weights in the presence of glutathione (1-3). Several studies have led to the suggestion that thioltransferase may play an important role in the maintenance of cellular protein thiol/disulfide status (4-6). In the past three decades, the purifications and characterizations of thioltransferases from different sources were reported (7-12).

Pig liver thioltransferase (PLTT), purified to homogeneity (12), consists of a single peptide of 105 amino acids with a calculated molecular weight of 11,740. Its primary structure was determined by amino acid sequence analysis which also indicated that the N-terminus of the protein was blocked by an acetyl group (13). Cysteine-22, which has an unusually low sulfhydryl pk_a value of 3.8, was proposed to be the active site of the enzyme by means of studying the reaction of thioltransferase with iodo[1-^{14}C]acetic acid (14) and inhibition studies with iodoacetamide (15). Polyclonal antibodies against PLTT were raised in a rabbit, and were shown to cross-react equally with calf liver thioltransferase and calf thymus glutaredoxin as well as with the pig liver enzyme (16). The immunological evidence, amino acid sequence homology, and catalytic properties (16-19) support the conclusion that thioltransferase (1) and glutaredoxin (18) of mammalian origin are two names given historically to the same protein.

Recently, we isolated the cDNA for PLTT by using the antibody described above to screen a pig liver cDNA library in lambda-gt11 and sequenced it (20). The cDNA contains 635 base pairs and its deduced amino acid sequence is the same as that directly determined (13), except that

the N-terminus was N-acetylalanine followed by glutamine rather than the reverse as originally concluded on the basis of an equivocal mass spectrometric interpretation.

In this chapter we report the high-level expression of PLTT in the unfused state, with full activity, in *E. coli*, and describe its purification and characterization.

EXPERIMENTAL PROCEDURES

Materials-- Expression vector pKK233-2 and its host cell E. coli JM105 were from Pharmacia; polynucleotide kinase, T4-DNA ligase, Klenow enzyme and restriction endonucleases were purchased from Boehringer Mannheim Biochemicals; PLTT was purified to homogeneity as reported (12); rabbit antiserum against PLTT was prepared as previously described (15); reagents for immunoblotting were from Bio-Rad and other standard reagents used in this work were obtained from Sigma, Bio-Rad, Difco Laboratories and Bethesda Research Laboratories.

Construction of PLTT Expression Vector-- Expression vector, plasmid pKK233-2, has a trc promoter induced by isopropyl-ß-D-thiogalactopyranoside (IPTG), a ribosome binding site (RBS) and an initiation codon, ATG, located within an unique NcoI site, CCATGG (21). A M13mp18 clone containing the whole length PLTT cDNA, which lacks RBS and NcoI sites at its translation initiation region, was isolated as previously described (20). In order to insert the cDNA into pKK233-2 between the NcoI and HindIII sites, we introduced a NcoI site at its initiation codon region by site-directed mutagenesis in which the first G of the original sequence, GCATGG, was replaced by a C. For this experiment, a 17mer oligonucleotide mutagenic primer 3'-dCGGACAGTGGTACCGAG-5', complementary to the original cDNA sequence of 5'-dGCCTGTCAGCATGGCTC-3' except the underlined G/G pair, was synthesized by an Applied Biosystems Model 380B DNA synthesizer. The site-directed mutagenesis was carried out according to Zoller and Smith (22) and the cDNA/M13mp18 single stranded DNA was used as a template. The mutant cDNA/M13mp18 clone containing the introduced NcoI site was verified by digestion of the clone with NcoI and HindIII. The NcoI-HindIII fragment

having the coding frame (321 bp) and 3'-noncoding region (330 bp) was ligated between the NcoI and HindIII sites of pKK233-2. The resulting PLTT expression vector, pTT1, was transformed into E. coli JM105. As a negative control, pKK233-2 alone was also transformed into JM105. The cloning steps described above were based on standard published procedures (23).

Expression and purification of PLTT-- The transformed JM105, cultured overnight at 37°C, was diluted 1 to 100 in 2 ml of fresh LB medium with 50 ug/ml of ampicillin and shaken at 37°C until an A_{550} of 0.4-0.7 was reached. At this time, IPTG was added to a final concentration of 1.5 mM, and the culture was continuously incubated for up to 8 h at the same temperature. Equal numbers of cells were removed at various times to monitor the rate of PLTT induction.

For a typical isolation of the expressed PLTT, cells cultured in 500 ml of LB medium as described above were harvested after 6 h, the optimal time of culture, by centrifugation at 15,000 x g for 10 min and the pellets were resuspended in 15 ml of a solution containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM dithiolthreitol (DTT) 0.5mM phenylmethylsulfonyl fluoride (PMSF), 400 µg/ml of egg white lysozyme, and 10% sucrose, at 4°C. The cells were disrupted by sonication (Heat Systems Ultrasonic,Inc., model W-220F, scale 5 for 5 x 30 s intervals) of the suspension at 4°C and the cell debris was sedimented at 25,000 x g for 30 min. The next two steps, heating and ammonium sulfate fractionation, followed the standard protocol (24) except that the first and second ammonium sulfate concentrations were 35% and 85%, respectively. The pellet from the 85% ammonium sulfate precipitation was dissolved in 2.5 ml of 10 mM sodium phosphate buffer, pH 6.5 (buffer A) containing 2mM DTT and subjected to

Sephadex G-100 gel filtration (2.5 x 36 cm) and elution with buffer A. The active fractions (46 ml) were incubated with 10 mM hydroxyethyldisulfide (HED) for 30 min at room temperature and loaded onto a CM-Sepharose column (2.5 x 26 cm), equilibrated with buffer A followed by washing with 150 ml of buffer A and 150 ml of 10 mM sodium phosphate buffer, pH 7.5 (buffer B). The bound disulfide enzyme form was eluted with a 200 ml linear salt gradient of 0.0 M to 1.0 M NaCl in buffer B, lyophilized and desalted on a Sephadex G-25 column (1.5 x 55) equilibrated with triple distilled water. The active fractions were pooled (20 ml), lyophilized, and the protein was dissolved in 1 ml of 50 mM sodium phosphate buffer, pH 7.5, containing 25% glycerol and stored at -70°C in a plastic vial. The protein concentration was determined by the BCA protein assay protocol according to the manufacture's direction (Pierce Chemical Co.) with bovine serum albumin as standard.

Thioltransferase activity assay-- The enzyme activity was assayed as described previously (24). Briefly, the reaction mixture contained 0.5 mM GSH, 1.4 units of glutathione reductase, 2.5 mM S-sulfocysteine (Cys-SO_3^-), 0.35 mM NADPH, 0.137 M sodium phosphate buffer, pH7.5, and the enzyme activity was monitored spectrophotometrically at 340 nm and 30°C. One unit of thioltransferase activity is defined as that amount of enzyme catalyzing the formation of 1 μmol of GSSG per minute under standard conditions. A blank reaction without enzyme was monitored simultaneously with each group of enzyme samples.

SDS-PAGE and Immunoblotting analysis-- The Laemmli system (25) was employed for routine SDS-PAGE analysis. Immunoblotting analysis was performed as previously described (20) in which rabbit antiserum, raised against PLTT, was used as the primary antibody and alkaline phosphatase

conjugated goat anti-rabbit IgG color reaction system was used to detect the immunoblotted bands.

Amino Acid Composition and N-terminal Sequence Analysis-- The homogeneous recombinant PLTT was carboxymethylated (26) and hydrolyzed in 6 N HCl for 24 h at 110°C in sealed vials. The amino acid composition was determined by the method of Cohen et al. (27) using a Beckman Model 121 Automatic Amino Acid Analyzer. The N-terminal sequence analysis was performed by automatic Edman degradation on an Applied Biosystems Model 477A pulse liquid protein/peptide sequencer.

Isoelectric Focusing-- Samples of thioltransferases were reduced by treating with 10 mM DTT or converted to the intramolecular disulfide form with 10 mM hydroxyethyl disulfide at room temperature for 30 min. The pI values were measured on a Servalyt Precote isoelectric focusing gel, pH 3-10, following the manufacturer instructions.

Optimum pH and Kinetic Behavior-- Activities of the expressed and native PLTT were assayed based on the same amount of protein (0.36 µg enzyme for each assay) and compared between pH 5.5 and 7.5 in 0.137 M sodium phosphate and between pH 8.0 and 10.0 in 0.137 M Tris-HCl. The thiol-disulfide exchange activity of the native and recombinant PLTT were studied as a function of Cys-SO_3^- concentration in the standard reaction mixture (24).

RESULTS

Construction of PLTT Expression Vector- The construction of the PLTT expression vector, pTT1, described under the Experimental Procedures, is summarized in Fig 8. For the purpose of cloning PLTT cDNA into pKK233-2, we changed the first G of GCATGG to a C at the translation initiation region of the cDNA in M13mp18 clone (Fig. 8A) by site-directed mutagenesis. The changed cDNA/M13mp18 clone (Fig. 8B) was confirmed by digestion with NcoI and HindIII, and the expected 650 base pair NcoI-HindIII fragment was observed on a 5% acrylamide gel (data not shown) which indicated that the NcoI site was successfully introduced into the cDNA at the appropriate location. The 650 bp PLTT cDNA fragment (Fig. 8C) was ligated to vector pKK233-2 (Fig. 8D) between the NcoI and HindIII sites. The newly constructed PLTT expression vector, pTT1, (Fig. 8E) contained the PLTT cDNA flanked upstream by a highly inducible trc promoter followed by a RBS and downstream by a strong rrnB transcription terminator adjacent to an ampicillin resistant gene.

Expression and Purification of PLTT-- E. coli JM105 competent cells were transformed with the newly constructed pTT1, and the expression of PLTT was induced by the addition of IPTG to the culture medium. During incubation, cells were withdrawn at different times after induction, diluted to the same absorbancy (A_{550}=0.58), and total cellular proteins were separated by SDS-PAGE (18%) and stained with Coomasie Brilliant Blue R-250 (Fig. 9, Panel A). The bands visualized at 11.7 kDa (solid arrow) and anticipated to be expressed PLTT were verified by immunoblotting analysis (Fig. 9, Panel B) with native PLTT as a positive control (Fig. 9, lane 10, Panel A, and lane 9, Panel B). The PLTT expression products were first detected after 1 h (lane 4. Panel A, and lane 3, Panel B) and reached

Fig. 8 Construction of PLTT expression vector-pTT1. The details of the construction of pTT1 are described in "Experimental Procedures". The black arch (or bar) in (A), (B), (C) and (E) represents the PLTT cDNA; the black triangle, hatched arch and open arch in (D) and (E) represent the trc promoter, transcription terminators-rrnBT_1T_2 and ampicillin resistant gene, respectively. (A) M13mp18 containing the original PLTT cDNA without the NcoI site; (B) M13mp18 containing the altered PLTT cDNA with the NcoI site; (C) isolated PLTT cDNA NcoI-HindIII fragment; (D) plasmid pKK233-2; (E) constructed PLTT expression vector pTT1. RBS, NcoI and HindIII sites are indicated.

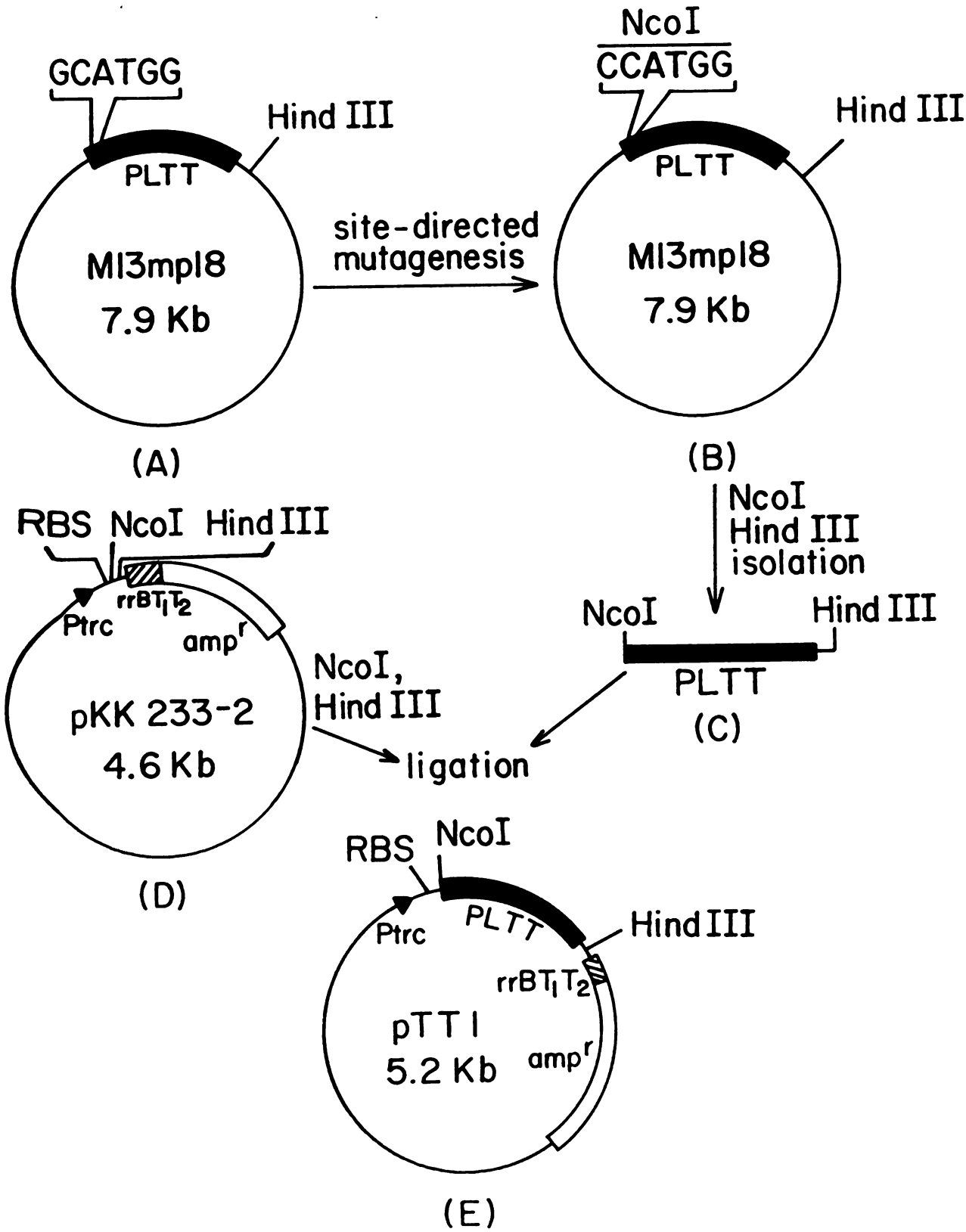
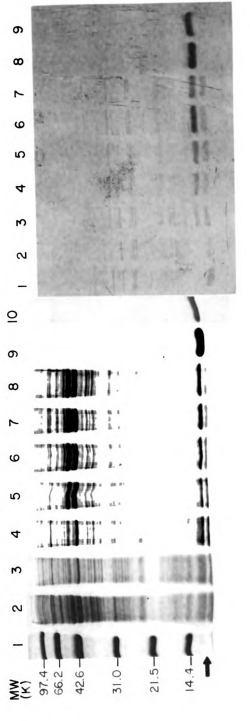

GCATGG
Hind III
PLTT
M13mp18
7.9 Kb
(A)
site-directed
mutagenesis
NcoI
CCATGG
Hind III
PLTT
M13mp18
7.9 Kb
(B)
NcoI
Hind III
isolation
NcoI
Hind III
PLTT
(C)
RBS NcoI Hind III
rrBT1T2
Ptrc
amp^r
pKK 233-2
4.6 Kb
(D)
NcoI,
Hind III
ligation
RBS
NcoI
Ptrc
PLTT
Hind III
rrBT1T2
pTT1
5.2 Kb
amp^r
(E)

Fig. 9 SDS-PAGE and immunoblotting analysis of the total cell proteins containing the expressed PLTT. Cells were withdrawn from cultures at different IPTG induction times, diluted to the same absorbance (A_{550}=0.58) and mixed 1:1 with SDS-PAGE loading buffer. Samples (20 µl) were analyzed by SDS-PAGE, stained with Coomassie Brilliant Blue (Panel A); or samples (10 µl) were immunoblotted and visualized with rabbit anti-PLTT (Panel B). Lane 1 of Panel A contained the Bio-Rad low molecular weight protein standards. Lanes A-2 and B-1 represents the control JM105/(pKK233-2) after 6 h induction; lanes A-3 and B-2, A-4 and B-3, A-5 and B-4, A-6 and B-5, A-7 and B-6, and A-8 and B-7 were JM105/(pTT1) representing 0 h, 1 h, 2 h, 4 h, 6 h and 8 h of induction, respectively; lanes A-9 and B-8 and lanes A-10 and B-9 were 5 µg and 0.5 µg of purified recombinant and native PLTT, respectively.


MW (K)
97.4
66.2
42.6
31.0
21.5
14.4
A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9

maximum levels after 6 h (lane 7, Panel A, and lane 6, Panel B). The JM105/pKK233-2 negative control (Fig. 9, lane 2, Panel A, and lane 1, Panel B) and the JM105/pTT1 sampled at zero time induction (Fig. 9, lane 3, Panel A, and lane 2, Panel B) showed no detectable thioltransferase by Western blot analysis. In 6 h, PLTT was overexpressed to the extent of 8% of the total soluble cell protein based on PLTT specific activity (Table III). The recombinant PLTT was purified to homogeneity as described in the "Experimental Procedures" section. Samples from various steps of the purification were analyzed by SDS-PAGE (18%) (Fig. 10) and summarized in Table III. The purified protein was a single band as judged by SDS-PAGE (Fig. 10) and gave a 56% yield of the total E coli thioltransferase-like activity.

Amino Acid Composition and N-terminal Sequence Analysis-- The molecular weight of native PLTT is calculated to be 11,740 based on the amino acid sequence (13) or the cDNA sequence (20). The absolute number of residues per mol for all amino acids present in the recombinant protein were analyzed and found to be identical with the theoretical number based on the amino acid sequence (Table IV).

The N-terminal sequence analysis indicated that the first five amino acid residues of the expressed enzyme were Ala-Gln-Ala-Phe-Val in agreement with that of the native enzyme as determined by PLTT cDNA sequence analysis (20). Of special interest was the finding that the expression product did not contain the pre-N-terminal methionine nor did an acetyl group appear at the N-terminus since the recombinant PLTT was expressed in E. coli where N-acetylation of proteins is not expected.

Isoelectric Focusing-- Isoelectric focusing gel analysis of the native and recombinant PLTTs revealed that the pI values compared between

TABLE III

Purification of recombinant pig liver thioltransferase

Recombinant pig liver thioltransferase was purified from sonicated transformed E. coli JM105 cells as described in EXPERIMENTAL METHODS. The cells were cultured in 500 ml of LB medium at 37°C and harvested after 6 h induction with 1.5 mM IPTG.

Steps	protein (mg)	TT* activity (units)	Specific Activity (units/mg)	Yield (%)	Purification Fold
Extract	180.1	1197	6.6	100	1.0
Heat and ammonium sulfate precipitation	41.0	725	17.7	60.5	2.7
Sephadex G-100	13.3	690	51.9	57.6	7.8
CM-Sepharose	8.2	672	82.0	56.1	12.4

* TT = Thioltransferase

Fig. 10 SDS-PAGE analysis of various steps in the purification of expressed PLTT. Each purification step was described under "Experimental Procedures". Samples of protein from various steps were loaded and analyzed by SDS-PAGE (18% acrylamide) (25). Proteins were visualized by the silver stain procedures of Guilian et al. (33). The samples were: 1.5 µg of cell extract (lane 1); 1.5 µg of ammonium sulfate precipitate (lane 2); 1.5 µg of Sephadex G-100 (lane 3), 0.5 µg of CM-Sepharose chromatography pool (lane 4); 0.5 µg of native PLTT (lane 5); lane 6 is a blank and lane 7 represents Bio-Rad low molecular weight protein standards. The solid arrow designates the mobility of native PLTT (11.7 kDa).

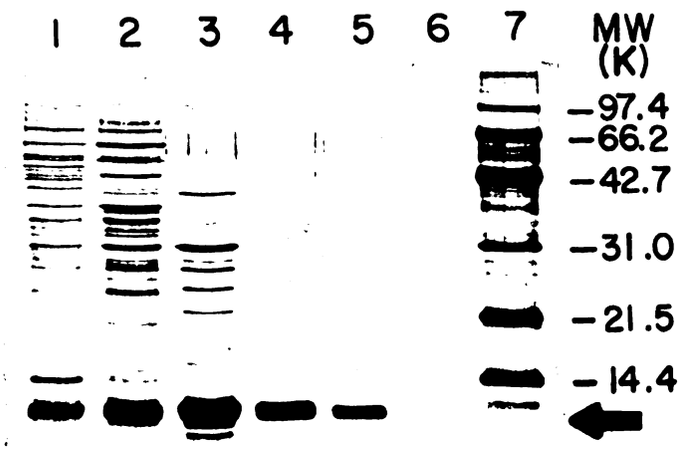

1
2
3
4
5
6
7
MW
(K)
97.4
66.2
42.7
31.0
21.5
14.4

TABLE IV

Amino acid composition of purified recombinant PLTT and comparison with native PLTT

Amino acid	Recombinant PLTT[a]	Native PLTT[b]
Asx	6.2	6
Glx	16.1	16
Ser	3.7	4
Gly	8.1	8
His	0.9	1
Arg	5.2	5
Thr	8.6	9
Ala	4.6	5
Pro	5.25	5
Tyr	1.0	1
Val	7.1	7
Met	1.0	1
Ile	7.1	7
Leu	12.3	12
Phe	6.1	6
Lys	1.8	2
Cys	3.7	4
Total	105	105

[a] Calculations were based on normalized concentration.

[b] The numbers of each amino acid were counted directly from the sequence of PLTT (12).

the reduced and oxidized forms and those between the two kinds of enzymes differed somewhat (Fig 11). As expected (12), the pI values of both oxidized forms were more basic than those of the reduced ones. However, the recombinant enzyme had a more alkaline pI than the native enzyme in either the reduced or oxidized state. The native PLTT had a pI of 6.1 and 7.6 for the reduced and oxidized form, repectively, whereas for the expressed enzyme, the corresponding values were 6.9 and 7.9. We attribute the pI differences between the native and the expressed enzymes to the lack of acetylation of the recombinant PLTT.

Optimum pH and Kinetic Behavior-- The activity of the recombinant PLTT was assayed over the pH range of 5.5-10.0 and was compared with that of the native enzyme (Fig. 12A). Both of the enzyme activities had an optimum at approximately pH 8.5, and their activity levels were equivalent based on the same quantity of protein (0.36 µg). As expected, the activities were extremely low at an acidic pH and significantly increased at lower hydrogen ion concentrations. In the standard assay system for thioltransferase, glutathione reductase is required to couple the reduction of glutathione disulfide (GSSG) to NADPH oxidation (24). Thus, in measuring the sensitivity of thioltransferase to hydrogen ion concentration, we took care to demonstrate that the standard assay contained enough glutathione reductase to detect thiol-disulfide exchange at each pH analyzed.

Thioltransferase activities of the native and recombinant enzymes, obtained in the standard assay in the presence of variable concentrations of Cys-SO_3^- and a constant amount of GSH (0.5 mM), were essentially identical (Fig. 12B). Both enzymes displayed non-Michaelis-Menten kinetics and the velocities decreased slightly at higher substrate

Fig. 11 Isoelectric focusing of PLTT. The pI values were measured on a Servalyt Precotes IEF gel, pH 1-10, according to the manufacture's instructions. The gel was stained with Coomassie Brilliant Blue. Lane 1, Serva pI marker proteins; lane 2, 4 µg of reduced recombinant PLTT treated with 10 mM DTT for 30 min at room temperature; lane 3, 4 µg of oxidized recombinant PLTT treated with 10 mM HED for 30 min at room temperature; lane 4, 4 µg of reduced native PLTT treated with 10 mM DTT; lane 5, 4 µg of reduced native PLTT treated with 10 mM HED.

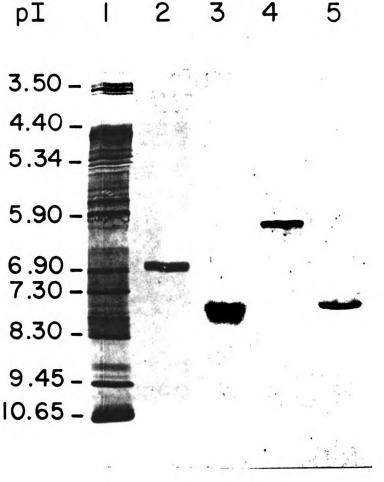
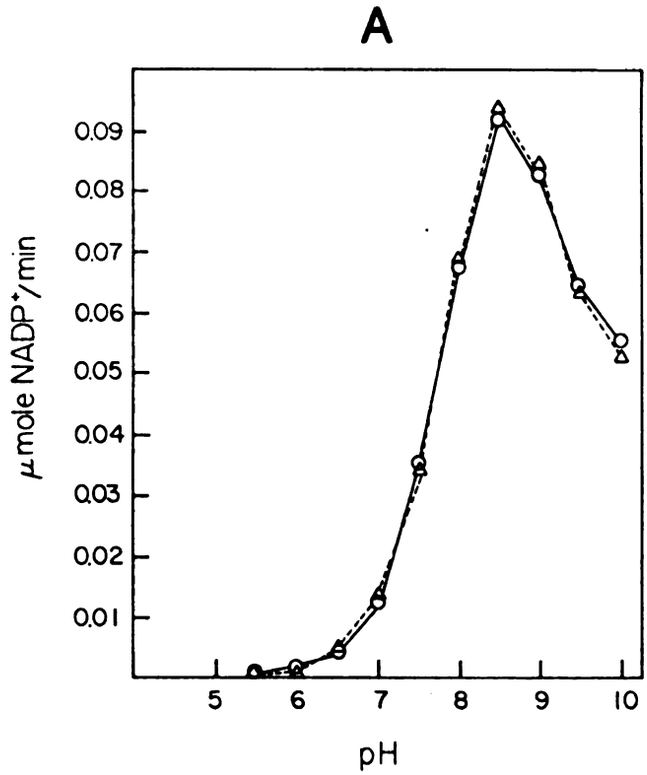
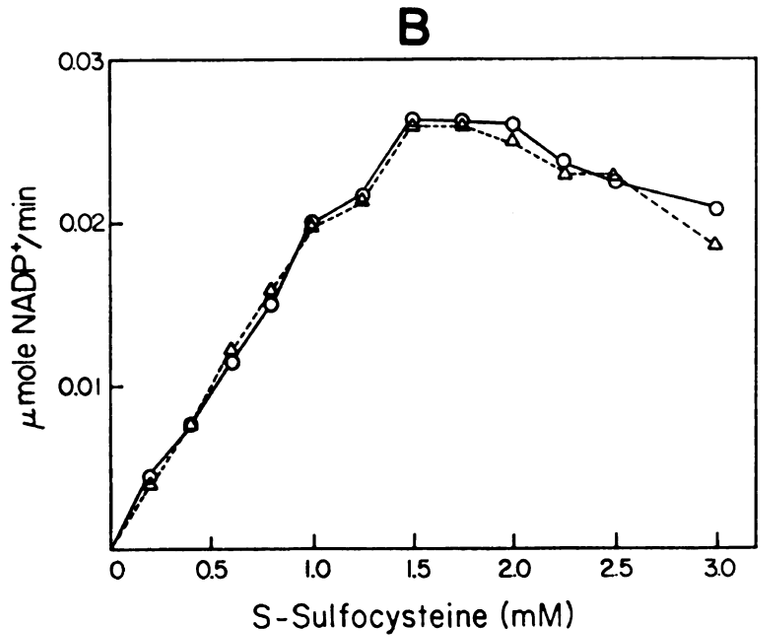

pI
1
2
3
4
5
3.50
4.40
5.34
5.90
6.90
7.30
8.30
9.45
10.65

Fig. 12 Comparison of the recombinant and native PLTT on optimum pH and kinetic behavior. PLTT activity dependence on pH (A) or on S-sulfocysteine concentration (B) was compared between the native (▲---▲) and recombinant (0---0) enzyme. A, The activity was measured by the standard assay system in which 0.137 M sodium phosphate (from pH 5.5 to 7.5) and 0.137 M Tris-HCl (from pH 8.0 to 10.0) were used. The activity is expressed as the net enzymatic velocity (formation of μmol $NADP^+$/min). B, The standard assay was used with increasing S-sulfocysteine concentration and constant amount of GSH (0.5 mM). Each assay contained 0.2 μg of either homogeneous native or recombinant enzyme. Each value is the average of two separate experiments.


A
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
μmole NADP+/min
5
6
7
8
9
10
pH


B
0.03
0.02
0.01
μmole NADP+/min
0
0.5
1.0
1.5
2.0
2.5
3.0
S-Sulfocysteine (mM)

concentrations. The $K_{0.5}$ for the two enzymes was estimated to be 0.6 mM.

DISCUSSION

The glutaredoxin gene of E. coli has been cloned and characterized in M13mp9 by Höög, et al. (28). The sequence of 255 base pairs comprising the glutaredoxin structural gene gave a deduced amino acid sequence identical to that determined previously (85 amino acids with an unblocked N-terminal methionine) (29). The coding region is preceded by two possible ribosome-binding sites and three possible promoters with -10 and -35 regions as judged by homology to consensus sequences. Glutaredoxin was amplified 100 fold in stain JM103[pEMBL9ECG] over that in wild-type E. coli cells.

Here, we report the first constuction of mammalian thioltransferase (glutaredoxin) expression system in E. coli. Plasmid pKK233-2 was designed for direct cloning and expression of eukaryotic genes lacking a prokaryotic RBS in the unfused state (21). This plasmid contains an efficient trc (trp-lac fusion) promoter, the lacZ RBS, and an ATG initiation codon within the unique NcoI site, CCATGG, which is common at the translational initiation region of eukaryotic genes (21, 30). In consideration of the requirement of a large amount of unfused PLTT for future structural and functional studies (see below), we selected pKK233-2 as our PLTT expression vector. But instead of CCATGG, a sequence of GCTAGG exists at the initiation site of PLTT cDNA (20) which also lacks prokaryotic RBS. For the purpose of inserting the cDNA into the plasmid between NcoI and HindIII sites, we changed the first G of GCATGG to a C by site-directed mutagenesis. The newly constructed vector, pTT1, specifically dependent upon IPTG induction, produced maximum levels of PLTT in E. coli JM105 grown in culture after 6 h of IPTG induction. A JM105/pKK233-2 strain was used as a negative control and no mammalian

thioltransferase band was detected by SDS-PAGE or Western-blot analysis even after 6 h of IPTG induction (Fig 9).

For purification of the expressed PLTT from the E. coli extract, we used a G-100 gel filtration step instead of consecutive Sephadex G-75 and G-50 gel filtrations as described previously (12). In addition, we eliminated the first CM-Sepharose chromatography step of the reduced enzyme (12) and directly applied the disulfide form of the expressed enzyme to a CM-Sepharose column. This simplified purification resulted in a high yield of 56% which represents 8% of the total E. coli soluble protein after 6 h induction. In contrast to the native PLTT which can be stored in the reduced form in water at -70^0 C without significant loss of activity, the recombinant enzyme aggregates to some extent under similar conditions. The recombinant enzyme can be stabilized in a 50 mM phosphate, pH 7.4, 25% glycerol solution at -70^0C.

To characterize the recombinant PLTT, it was subjected to amino acid composition analysis and N-terminal amino acid sequence analysis of the first five residues. Alanine was the N-terminus followed by Gln-Ala-Phe-Val in agreement with the sequence deduced from the cDNA sequence of PLTT for the first five residues (20). Methionine, which preceeds alanine as deduced from the cDNA sequence (20), was not found at the N-terminus of the recombinant protein, indicating its likely cleavage by the host cell. The N-terminal acetylation, caused by post-translational modification, found in all known mammalian thioltransferases (or glutaredoxins), e.g., PLTT (13), calf thymus glutaredoxin (31), rabbit bone marrow glutaredoxin (32), was absent in the recombinant enzyme. The function of acetylation of eukaryotic thioltransferases (glutaredoxins) at the N-terminus is not known, yet our results demonstrate that acetylation has little effect on

the kinetic properties of the corresponding pig liver enzymes. Since the only apparent difference between the recombinant and native proteins is the N-terminal acetylation of the latter, thereby blocking the positively charged amino group in the native protein, we conclude that this alteration most likely accounts for the more alkaline pI values of the recombinant PLTT.

The creation of a high-level of PLTT expression in the E. coli JM 105 system will provide the potential to produce large amounts of this enzyme for further kinetic, structural and functional studies. This expression system is also suitable for site-directed mutagenesis studies to establish unequivocal evidence for the active site cysteine residues and the influence of nearby basic amino acids on the pK_a of Cysteine 22 sulfhydryl group with respect to the mechanism of enzyme action.

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CHAPTER IV

IDENTIFICATION OF THE FUNCTIONAL AMINO ACIDS AT THE ACTIVE CENTER OF PIG LIVER THIOLTRANSFERASE BY SITE-DIRECTED MUTAGENESIS

SUMMARY

This report provides the first direct evidence for the identification of the essential amino acids at the catalytic center of a mammalian thioltransferase (glutaredoxin) using site-directed mutagenesis techniques. Seven mutagenic oligonucleotides were designed, synthesized, and used to exchange the original amino acids of Cys^{22} with Ser^{22}, Cys^{25} with Ser^{25}, Cys^{25} with Ala^{25}, Arg^{26} with Val^{26}, Lys^{27} with Gln^{27}, Arg^{26} Lys^{27} with Val^{26} Gln^{27}, and Cys^{18}-Cys^{82} with Ser^{18}-Ser^{82} by altering their codons in pig liver thioltransferase cDNA/M13mp18 clones. The seven mutant cDNAs, which contain the specific changes verified by sequencing analysis, as well as the wild-type cDNA (as a positive control) were subcloned into the expression vector, pKK233-2, between the unique NcoI and HindIII sites and expressed in E. coli JM105, separately, during 6 h of isopropyl-ß-D-thiogalactopyranoside induction. The soluble fraction of each cell extract containing the designed expression product was analyzed by SDS-PAGE and Western-blot which indicated that the proper protein was obtained. Each of the thioltransferases were purified to homogeneity and the enzyme activity of each mutant was measured and compared to that of the wild-type (defined as 100%) based on the same amount of protein (0.4 ug). The relative activity of Ser^{22}, Ser^{25}, Ala^{25}, Val^{26}, Gln^{27}, $Val^{26}Gln^{27}$, and Ser^{18}-Ser^{82} mutants were 0%, 110%, 9%, 32%, 67%, 5%, and 90%, respectively. These results indicated that the catalytic activity was the function of Cys^{22}, whereas the other original amino acids mentioned above differentially contributed to the enzyme activity. Unexpectedly, the replacement of

replacement of Cys with Ser at position 25 increased rather than decreased the enzyme activity, suggesting that the proposed intramolecular disulfide bond between Cys^{22} and Cys^{25} is not necessary for the catalytic mechanism of the Ser^{25} mutant, but does not rule out such a mechanism for the wild-type enzyme.

INTRODUCTION

Thioltransferase was originally called glutathione-homocystine transhydrogenase by Racker who discovered the enzyme in beef liver in 1955 (1). Glutaredoxin was first reported as a component in an alternate electron transport system for ribonucleotide reductase in mutant E. coli lacking thioredoxin (2). As more and more thioltransferases and glutaredoxins were purified and characterized from different sources (1-11), the similarities between the two proteins became obvious. First, both enzymes could catalyze the thiol-disulfide exchange reaction in the presence of GSH (1, 3, 10, 12). Second, amino acid sequence comparison of pig liver thioltransferase (13, 14), calf thymus glutaredoxin (15,16) and rabbit bone marrow glutaredoxin (11) demonstrated over 83% sequence homology among the three proteins, and all of them contained an active site sequence of -Cy^{22}-Pro-Tyr(Phe for the pig enzyme)-Cys^{25}-. Third, polyclonal antibodies raised against pig liver thioltransferase can recognize calf thymus glutaredoxin and calf liver thioltransferase with the same sensitivity (17), and the antiserum against calf thymus glutaredoxin cross-reacts with human placenta thioltransferase (18). These catalytic, structural, and immunological properties lead to the conclusion that thioltransferase and glutaredoxin are identical.

Pig liver thioltransferase was extensively characterized with respect to its biochemical properties, primary structure and immunology in this laboratory (8,13,17). The enzyme contains 105 amino acids and has a molecular weight of 11,740 (8). Its primary structure was determined by both direct amino acid sequence (13) and nucleotide sequence (14). Cys^{22} was proposed as the active site of the enzyme (19) whose nucleophilic sulfhydryl group had a pKa of approximately 3.8

(19,20). But direct evidence for the pivotal role of Cys^{22} and the adjuvant roles of Arg^{26} and Lys^{27} in the reaction mechanism have not been established.

Recently, we reported the high-level expression of pig liver thioltransferase in E. coli in the unfused state with all activity and kinetic behavior analogous to the native enzyme (21). The established expression system is efficient and suitable for making the soluble low molecular weight foreign protein. In this chapter, we describe the creation of seven mutant thioltransferases (glutaredoxins) by site-directed mutagenesis, their expression in this system, their purification to homogeneity, and their relative thiol-disulfide exchange catalytic behavior.

EXPERIMENTAL PROCEDURES

Materials--The pig liver thioltransferase cDNA/M13mp18 clone with the introduced NcoI site at the initiation codon was constructed previously (21). The expression vector, pKK233-2, and its host, E. coli JM105, were from Pharmacia LKB Biotechnology Inc.; polynucleotide kinase, Klenow enzyme, T4 DNA ligase, and restriction endonucleases were purchased from Boehringer Biochemicals; reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad; other standard reagents used in this work were obtained from Sigma, Difco Laboratories, and Bethesda Research Laboratories.

Mutagenic Oligonucleotides--Pig liver thioltransferase has two pairs of cysteines in the sequences, Cys^{22}-Pro-Phe-Cys^{25} and Cys^{78}-Ile-Gly-Gly-Cys^{82}. Previously, the first pair was concluded to be the active site (19) since only Cys^{22} has an unusual sulfhydryl pKa (3.8) and prokaryotic glutaredoxin has only one pair, analogous to the first eukarytic sequence. To directly test this proposal and identify other essential amino acids at the active center, seven mutagenic oligonucleotides complementary to the specific regions of the original non-transcribed strand of thioltransferase cDNA (15), were synthesized by an Applied Biosystems Model 380B DNA synthesizer. Each oligonucleotide was designed to substitute one or two original amino acid residues, and the changes were Cys^{22} to Ser^{22}, Cys^{25} to Ser^{25} or Ala^{25}, Arg^{26} to Val^{26}, Lys^{27} to Gln^{27}, $Arg^{26}Lys^{27}$ to $Val^{26}Gln^{27}$, and Cys^{78}-Cys^{82} to Ser^{78}-Ser^{82}.

Site-directed Mutagenesis and Nucleotide Sequencing--The replacements of the original amino acids with the designed ones were performed by changing their codons in the cDNA sequence using the site-directed mutagenesis technique. These experiments were carried

out based on the method of Zoller and Smith (22). Briefly, the single stranded M13mp18 DNA containing the thioltransferase cDNA with the unique NcoI site (21) was used as a template, and each of the seven mutagenic oligonucleotides, as well as the universal 17-mer sequencing oligonucleotide used as primer, were incubated in 0.2 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$ and 0.1 M dithiothreitol (DTT) at 80°C for 5 min, and the template and primers were annealed at room temperature for 30 min. The complimentary DNA synthesis and ligation reactions were held at 15°C for 6-8 h in the presence of 0.1 mM dNTPs, 0.5 mM ATP, 2 units of Klenow enzyme, and 6 units of T4 DNA ligase. Then, 5 µl of the ligation mixture was used to transform E. coli JM101 cells and the positive plaques were screened with ^{32}P-labeled mutagenic oligonucleotide. The hybridization positive clones containing the mutant thioltransferase cDNA were verified by nucleotide sequencing using the method of Sanger (23).

Construction of Expression Vectors for Mutant Thioltransferase--The seven mutant cDNA/M13mp18 replicative form DNAs were isolated and digested with NcoI and HindIII, separately, and each of the NcoI-HindIII fragments, containing a specific mutant, was inserted into plasmid, pKK233-2, between the NcoI and HindIII sites. The resulting expression vectors, named pTT2 to pTT8 (Table II), were transformed into E. coli JM105 cells, separately. All the molecular cloning procedures described here followed that of Sambrook, et al (24).

Expression and Purification of the Mutant Thioltransferases--Each mutant thioltransferase was expressed as described previously (21). Briefly, an overnight culture of transformed JM105 cells was diluted 1 to 100 in 500 ml LB medium (50 µg of ampicillin per ml medium) and

incubated at 37°C until an A_{550} of 0.4-0.7 was reached. At this point, IPTG was added to a final concentration of 1.0 mM, and the culture was continuously shaken at 37°C for 6 h. The purification of the mutant enzymes was performed using the same procedures described in the purification of the wild-type enzyme (21) except that the pH of buffer A and buffer B was different for various mutants (Table II). The NaCl gradient used in buffer B was also changed from 0-1.0 M (21) to 0-0.5 M. The expression products were named ETT (expressed thioltransferase, wild-type), ETT-Ser^{22} (mutant with Ser at position 22), ETT-Ser^{25}, ETT-Ala^{25}, ETT-Val^{25}, ETT-Gln^{27}, ETT-$Val^{25}Gln^{27}$, and ETT-Ser^{18}-Ser^{22}. The mutants of ETT-Val^{25} and ETT-$Val^{25}Gln^{27}$ were further purified by HPLC on a C18 reversed-phase column.

Raising Polyclonal Antibodies--Polyclonal antibodies against expressed wild-type pig liver thioltransferase were raised by immunization of a New Zealand male rabbit. The homogeneous protein (2.0 mg) was emulsified with 1 ml of complete Freund's adjuvant and injected subcutaneously. On the 14th and 21st days after the first injection, injections were repeated with 1 mg of the enzyme in incomplete Freund's adjuvant. The antiserum was collected by the method of Carroll et al. (25).

SDS-PAGE and Western-blotting--During the purifications, the cell extract of each sample was separated on a 15% SDS-polyacrylamide gel (26) and then transferred onto a nitrocellulose filter. The immunoblotting was analyzed as described previously (21) in which the antibodies described above were used as primary antibody with 1 to 200 dilution and an alkaline phosphatase conjugated goat anti-rabbit IgG color reaction system was employed to visualize the immunoblotted

bands.

Thioltransferase Activity Comparison--The concentration of each purified protein was determined by the BCA protein assay protocol according to the manufacture's instruction (Pierce Chemical Co.) with bovine serum albumin as standard, and the homogeneity of each mutant was verified by SDS-PAGE. The thiol-disulfide exchange activity of wild-type and mutant thioltransferases was measured and compared to each other based on the same amount of protein. The standard thioltransferase activity assay system in our laboratory was employed, i.e. a 500 µl reaction mixture contained 0.4 µg of enzyme, 0.5 mM GSH, 1.4 units glutathione reductase, 0.137 M sodium phosphate buffer, pH7.5, 0.35 mM NADPH, and 2.5 mM S-sulfocysteine. The reaction was monitored spectrophotometrically at 340 nm at 30°C. One unit of thioltransferase activity is defined as the amount of enzyme catalyzing the formation of 1 µmol of GSSG per min under the standard conditions. A blank reaction without enzyme was measured simultaneously with each group of enzyme samples.

RESULTS

Mutagenic Oligonucleotides and Site-directed Mutagenesis-- Six of the seven oligonucleotides were designed for exchange of the amino acids located in the alleged active center. The seventh mutant replaced the 2nd pair of cysteines, absent in procaryotic glutaredoxin, with serine residues. The length of the oligonucleotides are different and dependent on the number of bases needed for change. Normally, the mutant bases are at least 8 residues from each end of the oligonucleotide (22). In our case, the oligonucleotides varied between 17-mer and 29-mer (Table V) in length. As summarized in Table V, each oligonucleotide was complimentary to the non-transcribed thioltransferase cDNA strand in specific regions, except those designed mutant bases (underlined). The orientations and locations of these sequences as well as the amino acid alterations are indicated in Table V.

The strategy of molecular cloning and site-directed mutagenesis was described in Experimental Procedures. The JM101 cells transformed with the ligation mixture were plated onto YT agar plates, and those plates with a plaque density of approximately 500 pfu per plate (85 mm) were overlapped with a piece of dry nitrocellulose filter for transferring the DNAs (22, 23). To screen the mutants, each of the filters adsorbing specific mutant thioltransferase cDNAs was hybridized overnight with the corresponding ^{32}P-labeled oligonucleotide at room temperature. After washing at room temperature with 6 x SSC, the filters were washed at higher temperatures for 10 min; 65°C for oligonucleotide (oligo) 1 and 7, 50°C for oligo 2, 3, and 5, 54°C for oligo 4, and 60°C for oligo 6. There were several positive hybridization signals on each filter after

Table V
Oligonucleotide primers used in site-directed mutagenesis
of pig liver thioltransferase (PLTT)

The mutagenic oligonucleotide primers were designed and synthesized complementary to the specific regions (in parentheses) of the original non-transcribed PLTT cDNA sequence, except those underlined bases.

	Sequence of oligonucleotide primer (3' to 5')[a]	Location in PLTT cDNA[b]	Amino acid substitution
1.	TCGGGTGGTCAGGGAAGAC (AGCCCACCTGCCCCTTCTG)	82 to 100	Cys^{22} to Ser^{22}
2.	CGGGGAAGTCGTCTTTC (GCCCCTTCTGCAGAAAG)	91 to 107	Cys^{25} to Ser^{25}
3.	CGGGGAAGCGGTCTTTCT (GCCCCTTCTGCAGAAAGA)	91 to 108	Cys^{25} to Ala^{25}
4.	GGAAGACGCATTTCTGTG (CCTTCTGCAGAAAGACAC)	94 to 111	Arg^{26} to Val^{26}
5.	AGACGTCTGTCTGTGTC (TCTGCAGAAAGACACAG)	97 to 113	Lys^{27} to Gln^{27}
6.	GGAAGACGCATGTCTGTGTC (CCTTCTGCAGAAAGACACAG)	94 to 113	$Arg^{26}Lys^{27}$ to $Val^{26}Gln^{27}$
7.	CATTTCTCTCATATCCACCTTCGTGACTA (GTAAAGAGTGTATAGGTGGATGCACTGAT)	249 to 268	$Cys^{78}Cys^{82}$ to $Ser^{78}Ser^{82}$

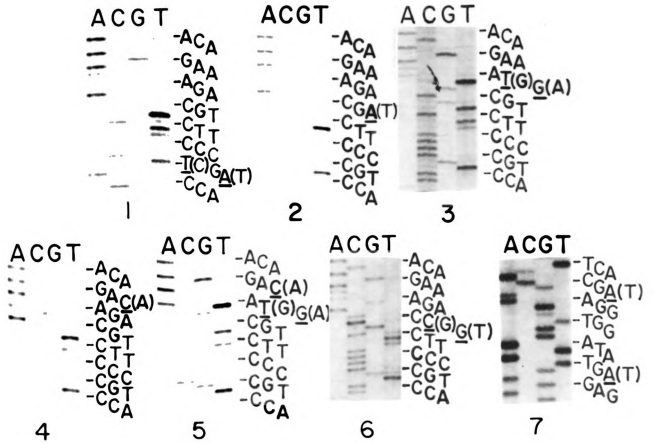
a The codons encoding the original amino acids are highlighted, and the orientations of the original non-transcribed cDNA strand (in parentheses) are from 5' to 3'

b The locations refers to the original sequences in PLTT cDNA complementary to the relative oligonucleotide primers.

autoradiography (data not shown). The mutant plaques were picked up and their DNAs were prepared (24). The base changes of each mutant were confirmed by nucleotide sequencing in which each of the mutant cDNAs in M13mp18s was used as template (Fig. 13). The mutant bases are underlined followed by the original bases in parentheses. From bottom to top is the direction of 5' to 3' of the sequence. Panel 1 to panel 7 display the codon alterations of TGC (Cys^{22}) to AGT (Ser^{22}), TGC (Cys) to AGC (Ser^{25}), AGA (Arg^{26}) to GTA (Val^{26}), AAG (Lys^{27}) to CAG (Gln^{27}), AGAAAG ($Arg^{26}Lys^{27}$) to GTACAG ($Val^{26}Gln^{27}$), TGC (Cys^{25}) to GCC (Ala^{25}), and TGT TGC (Cys^{18}-Cys^{22}) to AGT-AGC(Ser^{18}-Ser^{22}), respectively. The data shown here verify that the desired mutant thioltransferase cDNAs were obtained.

Expression and Purification of Mutant Thioltransferase--The seven mutant pig liver thioltransferase cDNAs of confirmed nucleotide sequence were subcloned into plasmid pKK233-2 between the unique NcoI and HindIII sites, separately. The seven newly constructed expression vectors, containing specific mutagenized thioltransferase cDNAs, were sequentially named pTT2 to pTT8 corresponding to the mutant protein products of ETT-Ser^{22}, ETT-Ser^{25}, ETT-Ala^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, and ETT-Ser^{18}-Ser^{22}. The expression vector for the wild-type enzyme (ETT) was named pTT1 previously (21). JM105 cells were transformed with the expression vectors, pTT1 to pTT8, separately, and the wild-type and mutant thioltransferases were expressed during 6 h of IPTG induction. The cell extracts were prepared as described before (21), and equal amounts of protein from each sample extract were analyzed by SDS-PAGE and by Western-blotting technique using antibodies against recombinant pig liver thioltransferase

Fig. 13 Nucleotide sequencing of the mutant cDNAs. Site-directed mutagenesis was performed by using each of the seven mutagenic oligonucleotides as a primer and the single-stranded M13mp18 DNA containing the thioltransferase cDNA as a template. The designed base substitutions were verified by nucleotide sequencing (dideoxy chain-termination method), in which each of the mutant cDNAs in M13mp18 was used as a template. The altered bases are underlined and followed by the original bases in the parentheses. Panels 1 to 7 show the codon changes for Ser^{22} (AGT), Ser^{25} (AGC), Val^{26} (GTA), Gln^{27} (CAG), $Val^{26}Gln^{27}$ (GTACAG), Ala^{25} (GCC), and Ser^{78}-Ser^{82} (AGT AGC), respectively.


ACGT
ACGT
ACGT
ACGT
ACGT
ACGT
ACGT
1
2
3
4
5
6
7

(Fig. 14). Panel A shows the patterns of crude cell extracts separated by SDS-PAGE stained with Coomassie Brilliant Blue R-250, whereas Panel B displayed the immunoblotting result visualized by the conjugated alkaline phosphatase color developing system. Lane 1 of panel A was Bio-Rad low molecular weight protein standards, lanes 2 to 9 of Panel A, or lanes 1 to 8 of Panel B were cell extracts containing ETT, ETT-Ser^{22}, ETT-Ser^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, ETT-Ala^{25}, and ETT-Ser^{18}-Ser^{22}, respectively. The position of thioltransferase was indicated with an arrow. The results clearly demonstrated that the wild-type and mutant type pig liver thioltransferases were successfully expressed in E. coli with approximately equal efficiency.

For purification of each mutant thioltransferase, the simplified method (21) was employed with some necessary modifications dependent on the pI value of the individual enzyme. The major change was the pH of buffer A and buffer B used in the carboxymethyl Sepharose step in the isolation process (Table VI). Since the ETT-Ser^{22} mutant had no detectable enzyme activity, it was purified blindly by following the same steps of purification as for ETT. ETT-Val^{26} and ETT-$Val^{26}Gln^{27}$ were further purified by HPLC with a C18 reversed-phase column. All mutant enzymes were purified to homogeneity, as seen by a single band on SDS-PAGE (Fig. 15). Lane 1 to lane 9 were loaded with Bio-Rad low molecular weight protein standards, ETT, ETT-Ser^{22}, ETT-Ser^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, ETT-Ala^{25}, and ETT-Ser^{18}-Ser^{22}, respectively.

Thioltransferase Activity Comparison--The enzyme activities of wild-type and mutant thioltransferases were measured by the standard thiol-disulfide exchange assay system (see Experimental Procedures) and were compared to each other based on the same amount of protein (0.4

Fig. 14 SDS-PAGE and immunoblotting analysis of the expressed mutant pig liver thioltransferase in cell extracts. The confirmed mutant cDNAs were separately subcloned into the expression vector, pKK233-2, and expressed in E. coli JM105 after induction with IPTG (6h). Cell extracts were obtained by sonicating the cells and sedimenting the cell debris. The same amount of total protein from each extract was analyzed by SDS-PAGE, stained with Coomassie Brilliant Blue (Panel A, 10 μg), or by immunoblotting visualized with rabbit anti-recombinant pig liver thioltransferase (Panel B, 1.0 μg). Lane A1, Bio-rad low molecular weight protein standards; Lanes A2 (B1)-Lanes A9 (B8) were samples containing ETT, ETT-Ser^{22}, ETT-Ser^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, ETT-Ala^{25}, and ETT-Ser^{78}-Ser^{82}, respectively. The arrow indicates the position of thioltransferase (glutaredoxin).

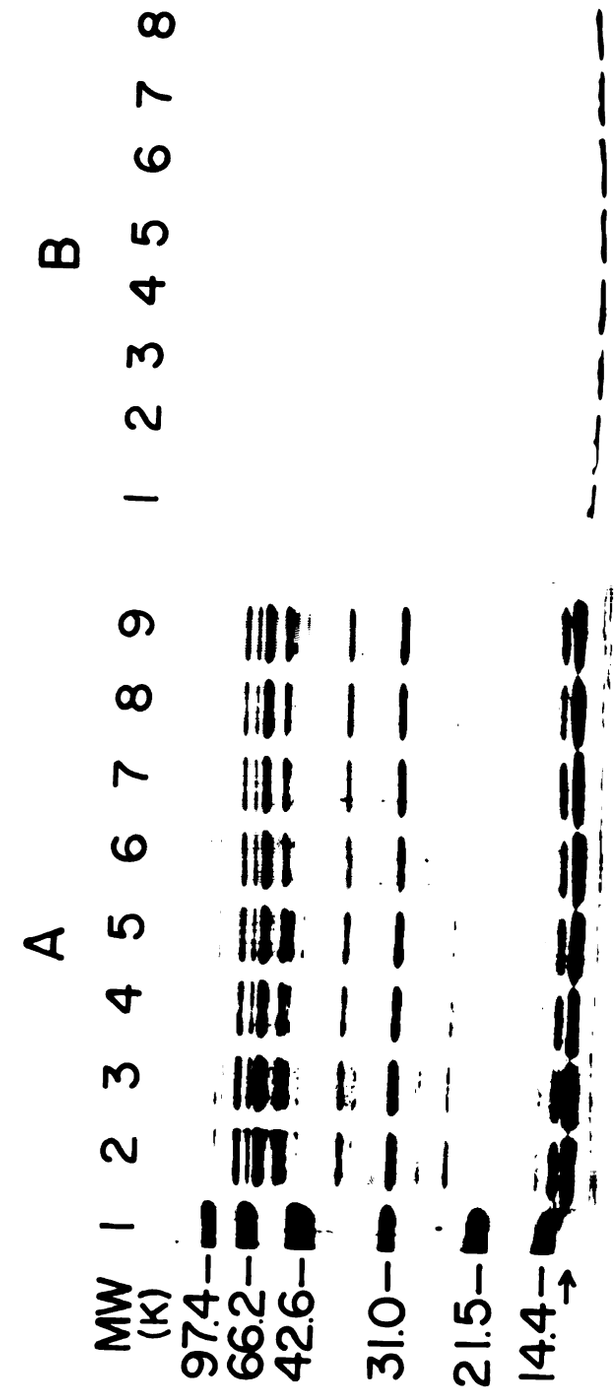

A
1 2 3 4 5 6 7 8 9
MW (K)
97.4
66.2
42.6
31.0
21.5
14.4
B
1 2 3 4 5 6 7 8

TABLE VI

Purifications of wild-type and

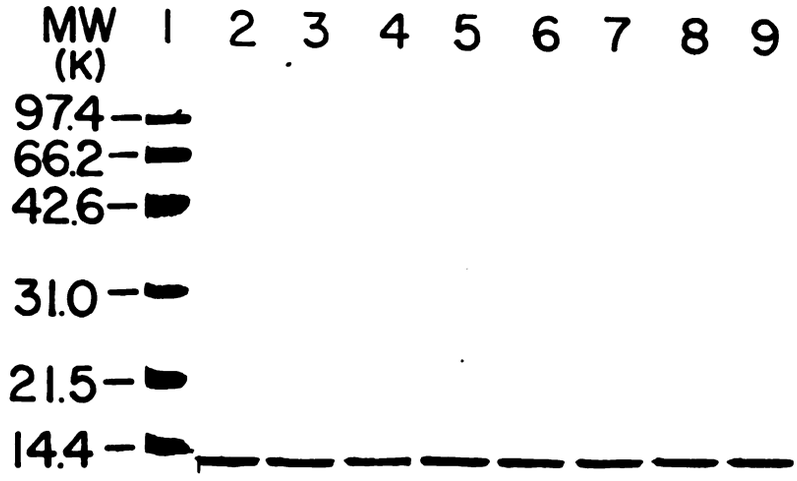
mutant thioltransferase by CM

Sepharose chromatography

The mutagenic oligonucleotides, numbered from 1 to 7, are the same as those in Table I, and their corresponding expression vectors are named from pTT2 to pTT8, respectively. The mutant products and the buffer pH used in purification are listed. Buffer A and Buffer B, used in the carboxymethyl Sepharose chromatography step, are 20 mM sodium phosphate at the pH shown. The vector pTT1 expresses the wild-type thioltransferase.

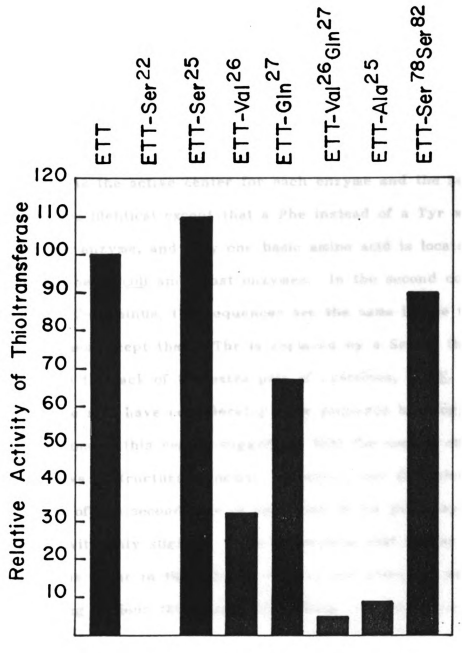
Oligo Number	Vector Name	Protein Product	Buffer A pH	Buffer B pH
0	pTT1	ETT	6.5	7.5
1	pTT2	ETT-Ser^{22}	6.5	7.5
2	pTT3	ETT-Ser^{25}	6.2	7.1
3	pTT4	ETT-Ala^{25}	6.2	7.2
4	pTT5	ETT-Val^{26}	6.0	7.0
5	pTT6	ETT-Gln^{27}	6.0	7.0
6	pTT7	ETT-$Val^{26}Gln^{27}$	5.0	6.0
7	pTT8	ETT-Ser^{78}-Ser^{82}	6.5	7.5

Fig. 15 SDS-PAGE analysis of the purified mutant pig liver thioltransferases. Each of the mutant thioltransferases was purified as described in Experimental Procedures. The purified samples (1 μg) were analyzed on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Lanes 1 to 9 were Bio-Rad low molecular weight protein standards, ETT, ETT-Ser^{22}, ETT-Ser^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, ETT-Ala^{25}, and ETT-Ser^{18}-Ser^{22}, respectively.


MW
(K)
1
2
3
4
5
6
7
8
9
97.4
66.2
42.6
31.0
21.5
14.4

μg) (Fig. 16). The activity of the wild-type thioltransferase (ETT) was defined as 100%, and accordingly, the relative activities of the mutants were 0% for ETT-Ser^{22}, 110% for ETT-Ser^{25}, 32% for ETT-Val^{26}, 67% for ETT-Gln^{27}, 5% for ETT-Val^{26}Gln^{27}, 9% for ETT-Ala^{25}, and 90% for ETT-Ser^{78}-Ser^{82}. As speculated, exchange of Cys with a Ser at position 22 of pig liver thioltransferase completely eliminated the enzyme activity. This is the first direct evidence revealing that Cys^{22} is the required active site amino acid residue of mammalian thioltransferase (glutaredoxin). It has been known that the sulfhydryl group of Cys^{22} has an extremely low pK_a of 3.8 (20), and this property has been speculated to be facilitated by the two neighboring amino acids, Arg^{26} and Lys^{27}. Changing the Arg with a Val or the Lys with a Gln at the active center, we found a 68% or a 33% activity loss, respectively. But if the two basic amino acids were changed simultaneously, only 5% of the wild-type enzyme activity remained. These results indicated that both basic amino acids strongly influenced the activity of the native enzyme. Interestingly, the replacement of Cys with a Ser at position 25 caused an increase rather than a decrease in enzyme activity, suggesting that the formation of an intramolecular disulfide bond between Cys^{22} and Cys^{25} is not the only possible mechanism for enzymatic catalysis. However, the substitution of the Cys with an Ala at this position caused a 91% activity loss, implying that an amino acid residue with a more polar side group, such as $-CH_2SH$ or $-CH_2OH$, at this position is required for optimal enzyme activity. The second pair of cysteines, Cys^{78} and Cys^{82}, near the C-terminus of the enzyme, can be substituted with serines without altering enzyme activity more than 10%.

Fig. 16. Enzyme activity comparison of the mutant and wild-type pig liver thioltransferases. Using the same amount of protein (0.4 ug), the thioltransferase activity of each purified enzyme was measured by the standard assay system which contained 0.5 mM GSH, 1.4 units of glutathione reductase, 2.5 mM S-sulfocysteine, 0.35 mM NADPH, and 137 mM sodium phosphate buffer, pH 7.5. The relative activity of mutant thioltransferases as based on the wild-type enzyme activity that was defined as 100%.

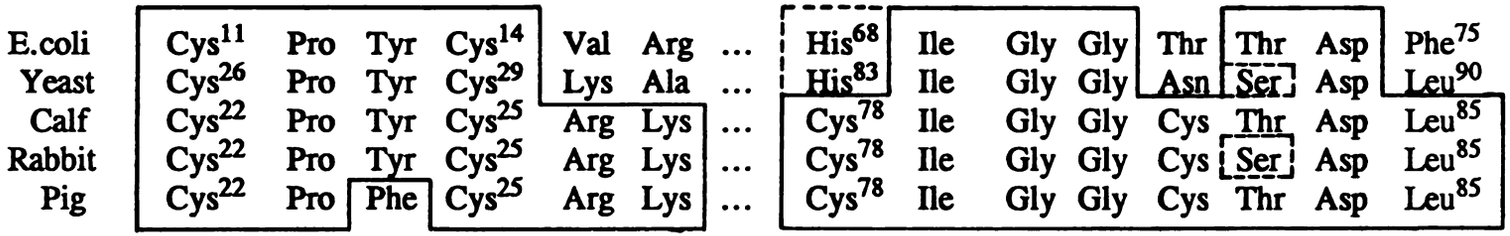

Relative Activity of Thioltransferase
120
110
100
90
80
70
60
50
40
30
20
10
ETT
ETT-Ser22
ETT-Ser25
ETT-Val26
ETT-Gln27
ETT-Val26Gln27
ETT-Ala25
ETT-Ser78Ser82

DISCUSSIONS

The primary structure of thioltransferase (glutaredoxin) has been reported for E. coli (27), calf thymus (15, 16), pig liver (13, 14), rabbit bone marrow (11), and recently yeast (28). All the proteins have an active site of Cys-Pro-Tyr(Phe for pig enzyme)-Cys-, while the three mammalian enzymes contain an additional pair of cysteines near the C-terminus (Fig. 17). The sequences of the two regions containing the cysteine pairs are highly conserved in thioltransferases (glutaredoxins). The first region is the active center for each enzyme and the sequences in this region are identical except that a Phe instead of a Tyr was found in the pig enzyme, and only one basic amino acid is located in this region for the E. coli and yeast enzymes. In the second conserved region near the C-terminus, the sequences are the same in the three mammalian enzymes except that a Thr is replaced by a Ser in the rabbit enzyme. Despite the lack of the extra pair of cysteines, the E. coli and the yeast enzyme still have considerably high sequence homology to the mammalian proteins in this region suggesting that the second conserved region might have a structural function. However, our data showed that the replacement of the second pair of cysteines in the pig enzyme affected its activity only slightly. It is interesting that similar cysteine pair distributions occur in thioredoxin, another low molecular weight protein catalyzing various thiol-disulfide exchange reactions, i.e. there are two pair of cysteines in mammalian enzymes and only one pair in the active center of the bacterial and yeast thioredoxins (29,30).

The present work provides the first direct evidence for the identification of the essential amino acids in the active center of

Fig. 17 Comparison of amino acid sequences in two conserved regions. The amino acid sequences of thioltransferases from E. coli, yeast, calf thymus, rabbit bone marrow, and pig liver are compared in two cysteine containing regions. Alignment is based on the active site and the locations of the two regions are indicated with the number for each enzyme. The sequence between the two regions is omitted. Identical amino acids are outlined by solid lines, whereas dashed lines denote differences.


E.coli Cys11 Pro Tyr Cys14 Val Arg ... His68 Ile Gly Gly Thr Thr Asp Phe75
Yeast Cys26 Pro Tyr Cys29 Lys Ala ... His83 Ile Gly Gly Asn Ser Asp Leu90
Calf Cys22 Pro Tyr Cys25 Arg Lys ... Cys78 Ile Gly Gly Cys Thr Asp Leu85
Rabbit Cys22 Pro Tyr Cys25 Arg Lys ... Cys78 Ile Gly Gly Cys Ser Asp Leu85
Pig Cys22 Pro Phe Cys25 Arg Lys ... Cys78 Ile Gly Gly Cys Thr Asp Leu85

mammalian thioltransferases (glutaredoxins). Based on the proposed active site, we originally designed and synthesized six mutagenic oligonucleotides (Table I, oligo 1, 2,and 4-7) to create six mutants by site-directed mutagenesis. The surprising result from the ETT-Ser^{25} mutant prompted us to create a seventh mutant, ETT-Ala^{25}. The substitution of certain original amino acids caused large pI shifts, thus the pH of the buffers used in the purifications had to be modified (Table II). The pK_a value of the sulfhydryl group of Cys^{22} (pK_a = 3.8) is much lower than that of normal cysteine (pK_a = 8.5±0.5) (31), and Cys^{22} was speculated to be the active site of thioltransferase (19). This was directly confirmed by results of changing Cys^{22} to Ser^{22}, which totally eliminated the enzyme activity. These data also showed that the amino acids, Arg^{26}, and Lys^{27} are required for optimal enzyme activity since exchange at any of these positions generally decreased the activity with the exception that replacement of Cys^{25} with Ser^{25} increased rather than decreased the enzyme activity. This discovery necessitated a reevaluation of the enzyme mechanism for the mutant enzymes not capable of forming an intramolecular disulfide. Individual replacement of Arg^{26} or Lys^{27} with Val^{26} or Gln^{27}, led to a relative 32% or 67% enzyme activity, respectively, but altering the two basic residues together with two neutral amino acids caused a cooperative loss in activity. How the two basic amino acids influence the catalytic activity was addressed in a companion paper (32).

We did not try to change the two amino acids, Pro and Phe, between the two cysteines at the active center. However, two similar studies in T4 (33) and E. coli (34) thioredoxin were reported recently. Joelson, et al. (33) created three mutants, Cys-Gly-Pro-Cys, Cys-Val-Pro-

Cys, and Cys-Gly-Tyr-Cys, at the active site of T4 thioredoxin which has the sequence, Cys-Val-Tyr-Cys, and found no significant changes in the enzyme activity. Gleason et al. (34) constructed two mutants, Cys-Gly-Arg-Pro-Cys and Cys-Ala-Cys, at the native protein active site, Cys-Gly-Pro-Cys, of E. coli thioredoxin by altering the size, and demonstrated that the longer mutant lost 85% of its activity, whereas the one with a shorter chain had no activity. The mutation studies implied that the distance rather than the specific amino acids substituted between the two active site cysteines is important, and the 14 atom disulfide loop at the active site of the oxidized enzyme seems to have been the preferred choice during evolution. However, as we demonstrate here, a serine at position 25 might have been expected to enjoy an evolutionary advantage.

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CHAPTER V

CHARACTERIZATION OF MUTANT PIG LIVER THIOLTRANSFERASE

SUMMARY

Seven mutant pig liver thioltransferases (glutaredoxins) were created by site-directed mutagenesis (Yang and Wells (1991) J. Biol. Chem. Submitted for publication). The properties of these mutants with respect to structure and function were analyzed and compared with those of the wild-type enzyme. The pI of each protein was measured in its reduced and oxidized form by isoelectric focusing and the resulting pI shifts of the mutants relative to the wild-type were compared. The optimum pH for the enzyme activity of the mutants was determined to lie between pH 8.5-9.0, similar to that the wild-type enzyme. The kinetic behavior of the enzyme activity with respect to substrate, S-sulfocysteine, concentration with a constant amount of GSH was analyzed. In order to test the influence of the amino acid exchanges on the pK_a of the sulfhydryl groups of Cys^{22}, the active site of thioltransferase, the pK_a values for the mutant enzymes were obtained by studying the second order kinetics of the alkylation reaction of equal molar concentrations of iodoacetamide and thioltransferase. Conclusive evidence was obtained that Cys^{22} is the active site of the enzyme, and the extremely low pK_a value of its sulfhydryl group is facilitated primarily by Arg^{26}. In addition, the second pair of cysteines, Ser^{78} and Ser^{82}, near the C-terminus, are not directly involved in the active center but they may play a role in defining the native protein structure. It is possible that Cys^{25} is not necessary for enzyme activity, but an amino acid with the size and polarity, e.g., serine, is essential at this position. The role of Lys^{27} at the active center is different from that of Arg^{26}, and may be important in stabilizing the ES intermediate by electrostatically attracting negatively charged groups of substrates (e.g. $-COO^-$ or $-SO_3^-$

$-COO^-$ or $-SO_3^-$ of Cys-SO_3^- or the $-COO^-$s of GSH) with its positively charged side chain ($-NH_3^+$). Dehydroascorbate reductase activity of thioltransferase (glutaredoxin), (Wells et al. (1990) J. Biol. Chem. 265, 15361-15364) was measured for each mutant and compared with that of the wild-type enzyme revealing a 90% increased activity for the mutant, ETT-Ser^{25}. These results are consistent with the suggestion that the formation of an intramolecular disulfide bond between Cys^{22} and Cys^{25} is not required for enzyme activity of selected mutants.

INTRODUCTION

Thioltransferase (Glutaredoxin) is a low molecular weight heat stable protein with activity catalyzing thiol-disulfide exchange reactions in the presence of GSH. These reactions are considered important in maintaining cellular thiol/disulfide equilibria (1-3). There are four half-cystine residues in all sequenced mammalian thioltransferases, and two of them are located in a catalytic center near the N-terminus (4-8), whereas there are only two cysteine residues in yeast (9) and E. coli (10). The active site of thioredoxin from E. coli was identified to be Cys^{32} and to have a sulfhydryl pK_a value of 6.7, with high sensitivity to alkylating reagents (11). Like thioredoxin, when incubated with iodoacetic acid or iodoacetamide, thioltransferase was inhibited by alkylation at its active site, Cys^{22} (12). The pK_a of the Cys^{22} sulfhydryl group was estimated to be 3.8, much lower than a normal pK_a of cysteine (8.5±0.5)(13). The extremely acidic pK_a of the Cys^{22} thiol group is believed to be facilitated by its unique surrounding microenvironment, but which amino acid residues are involved in such actions is not established.

We reported the high-level expression of pig liver thioltransferase in E. coli, and showed that the recombinant enzyme was identical to the native one in most biochemical properties except the pI difference caused by the nonacetylated N-terminal alanine of the expressed protein (14). Recently, it was reported from this laboratory that thioltransferase, as well as protein disulfide isomerase, had intrinsic dehydroascorbate (DHA)[1] reductase activity and may be involved in the major mechanism for the regeneration of ascorbic acid from dehydroascorbic acid in animal cells (15). In Chapter IV (16), we

described the creation of seven mutant pig liver thioltransferases with amino acid substitutions in the active center by site-directed mutagenesis and identified the essential amino acids for thiol-disulfide exchange activity. Cys^{22} was directly identified as the essential catalytic site since a replacement with serine at this position totally eliminated the activity of the mutant protein; and Arg^{26} and Lys^{27} were required to maintain full enzyme activity. An increase in activity by the replacement of Cys^{25} with Ser^{25} led to the suggestion that the formation of an intramolecular disulfide bond between Cys^{22} and Cys^{25} was not necessary for the catalytic mechanism (16).

In this report, we evaluate the specific roles of the amino acids in the active center in the reaction mechanism by further characterization of the mutants including analysis of the pI values, kinetic behaviors, pH optima, pK_a of the Cys^{22} sulfhydryl group, and the DHA reductase activities.

EXPERIMENTAL PROCEDURES

Materials-- Isoelectric focusing gel and pI marker proteins were from Serva; dithiolthreitol (DTT), NADPH, glutathione reductase, and iodoacetamide were from Sigma; 2-hydroxyethyl disulfide (HED) was purchased from Aldrich Chemical Co. Inc.; S-sulfocysteine (Cys-SO_3^-) was prepared as described previously (17); Sephadex G-25 was from Pharmacia; dehydroascorbic acid was obtained from Fluka Chemika-Biochemika. All other standard reagents are analytical grade.

Preparation of Reduced thioltransferases-- The wild-type and mutant pig liver thioltransferases were expressed and purified to homogeneity as described previously (14, 16). Each of the purified enzymes was incubated with 25 mM DTT in the presence of 100 mM sodium phosphate buffer, pH 7.5 at room temperature for 30 min. The excess DTT and the salts were removed by passing the incubation mixture through a Sephadex G-25 gel filtration column (1.5 x 70 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.5. The reduced protein was eluted with the same buffer used to equilibrate the column and was concentrated in a centriprep 10 concentrator (Amicon) by centrifugation. Each of the concentrated reduced thioltransferases was then divided into aliquots of 0.1 ml and stored at -70°C until used.

Isoelectric Focusing Analysis of The Mutant Thioltransferases--The reduced form and oxidized form of pig liver thioltransferases were obtained by incubating 1 ug of each purified enzyme with 10 mM DTT and 10 mM HED, respectively, at room temperature for 30 min. The incubation mixtures (8 µl each) were then directly loaded onto a pre-cooled Servalyt Precote isoelectric focusing gel (125 x 125 mm), pH 3-10. The electrophoresis of the gel was performed according to the

manufacturer's instruction. Briefly, the gel was first run at a constant current of 5 mA until a power of 4 W was reached, then at a constant power of 4 W until 1700 volts were reached, and finally, the current remained at 1-2 mA for 10 min. The gel was fixed with a solution of 36% methanol, 6% trichloroacetic acid, and 3.6% sulfosalicylic acid, and was stained with Coomassie Brilliant Blue R-250.

Optimum pH of the Mutant Thioltransferases-- The thiol-disulfide exchange activity of the wild-type and the mutant pig liver thioltransferases was measured over the pH range of 5.5-9.5 in a 500 µl mixture containing 0.5 mM GSH, 1.4 unit glutathione reductase, 2.5 mM Cys-SO_3^-, 0.35 mM NADPH, 1.5 mM EDTA, 137 mM sodium phosphate buffer (pH 5.5-7.5) or 137 mM Tris-HCl buffer (pH 8.0-9.5). The amount of protein used in these experiments was different for each individual enzyme, depending on the relative catalytic activity detected.

Kinetic Behavior-- Thioltransferase activity dependence on the concentration of Cys-SO_3^- was determined by the standard enzyme assay system with various amount of the substrate, in 137 mM sodium phosphate buffer, pH 7.5, and 1.5 mM EDTA, 0.5 mM GSH, 0.35 mM NADPH and 1.4 units of glutathione reductase.

The pK_a Values of Mutant and Wild-type Thioltransferases-- Equal concentrations (60 µM) of the reduced thioltransferase, obtained as described above, and iodoacetamide were incubated in 100 mM sodium citrate buffer (pH 2.5-5.5) or 100 mM sodium phosphate buffer (pH 6.0-9.0) at room temperature. At different time points, various amounts of incubating mixture were withdrawn, and the enzyme activities were assayed after appropriate dilution, i.e., the quantity of each enzyme used for activity measurement was different. The alkylation reaction of

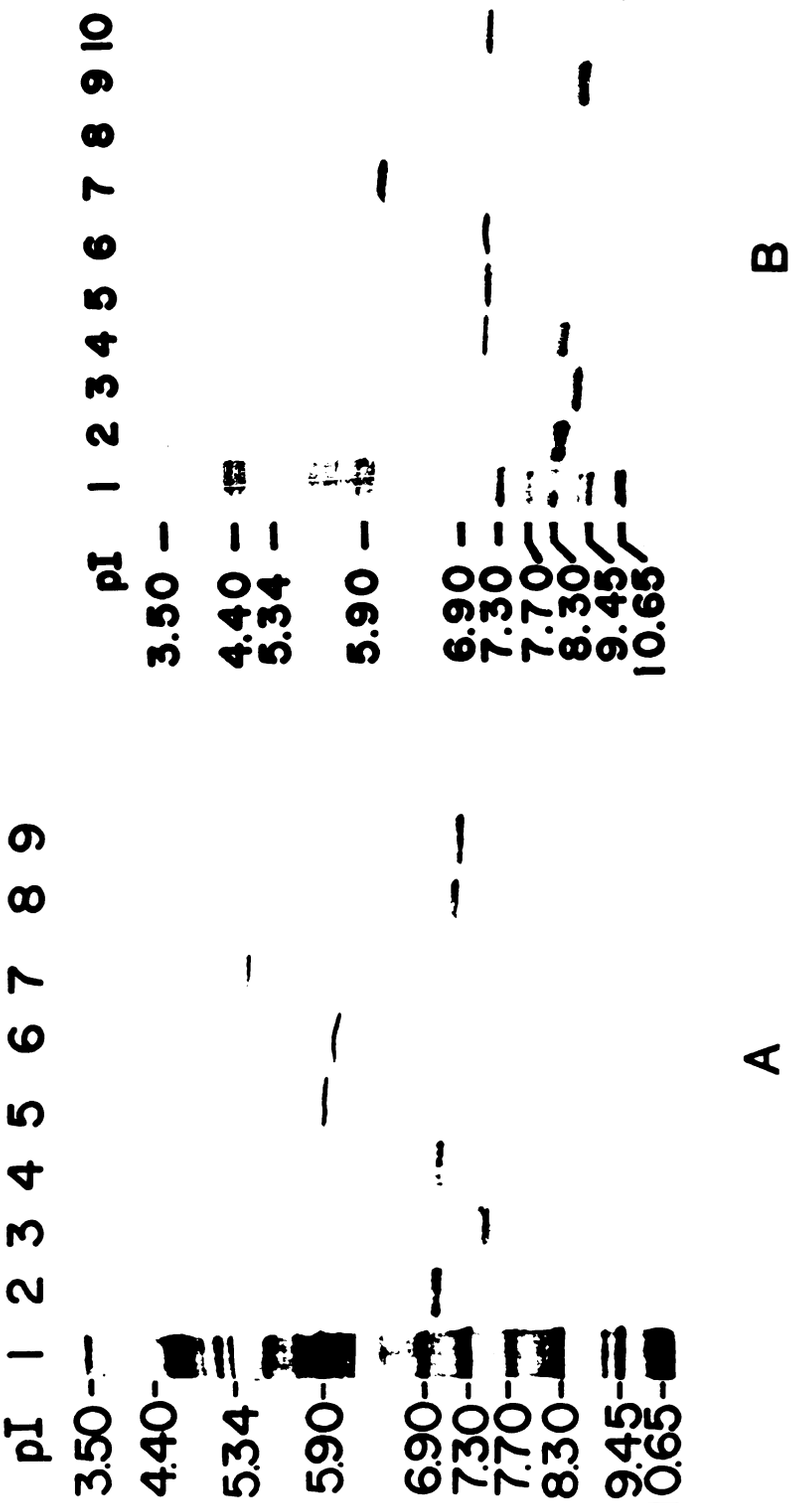
equal concentrations of thioltransferase and iodoacetamide followed second order reaction kinetics, and the apparent rate constant, k_{app}, of these reactions at each pH for each enzyme was determined. A plot of the dependence of k_{app} on pH gave an estimated pK_a value for Cys^{22} of each mutant retaining a cysteine residue at the 22 position.

The DHA Reductase Activity of Thioltransferase--The DHA reductase activity of the wild-type and each mutant thioltransferase was measured in a 500 µl mixture of 2 mM GSH, 1.5 mM DHA, 160 mM sodium phosphate buffer, pH 6.8, 1.2 mM EDTA, and 0.4 µg thioltransferase, spectrophotometrically, by monitoring the increase in absorbance at 265.5 nm and 30°C. One unit of DHA reductase activity is defined as that amount of enzyme catalyzing the formation of 1 nmol of ascorbic acid per min under the standard conditions. A blank control without enzyme was measured simultaneously with each group of activity assays.

RESULTS AND DISCUSSION

Isoelectric Focusing Analysis--The wild-type thioltransferase (ETT) and mutant thioltransferases (ETT-Ser^{22}, ETT-Ser^{25}, ETT-Ala^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, and ETT-Ser^{18}-Ser^{82}) were analyzed both in their reduced forms (Fig. 18A) and oxidized forms (Fig. 18B) by isoelectric focusing. In panel A, lanes 1 to 9 were loaded with Serva pI marker proteins, ETT, ETT-Ser^{22}, ETT-Ser^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, ETT-Ala^{25}, and ETT-Ser^{18}-Ser^{82}, respectively. In panel B, the pI of the oxidized forms of each protein are shown. Lanes 1 to 7 and lane 9 were loaded with the same enzyme as those in Panel A and lane 10 was ETT-Ala^{25}. As seen in Fig. 18, the pI values of the reduced thioltransferases are 7.0 for ETT, ETT-Ser^{25}, ETT-Ala^{25}, and ETT-Ser^{18}-Ser^{82}, 7.5 for ETT-Ser^{22}, 5.8 for ETT-Val^{26}, 6.1 for ETT-Gln^{27}, and 5.3 for ETT-$Val^{26}Gln^{27}$, whereas the pI values of the oxidized forms are approximately 8.0 for ETT, ETT-Ser^{25}, ETT-Ala^{25}, and ETT-Ser^{18}-Ser^{82}, 8.4 for ETT-Ser^{22}, 7.0 for ETT-Val^{26} and Gln^{27}, and 6.0 for ETT-Val^{26}-Gln^{27}. These results clearly indicated that exchange of some amino acids, especially the charged residues (e.g., Arg and Lys), at the active sites caused the pI shifts of the proteins. Normally, when a protein is in its native folded form, the pI value of the protein is the sum of the net charges on the surface of the molecule (18). In the present case, all thioltransferases, including the wild-type and mutants, analyzed on isoelectric focusing gel were in their native folded forms. Thus, the pI values of these proteins obtained by this technique should reflect the total surface charges of these native molecules and the pI shift should follow the changes of the surface charged groups. The substitution of Cys with a Ser at

Fig. 18. Isoelectric focusing of mutant thioltransferases. The purified wild-type and mutant thioltransferases (1 ug) were treated with 10 mM DTT (Panel A) or 10 mM HED (Panel B) for 30 min at room temperature, and the pI values were measured on a Servalyt Precotes IEF gel, pH 3-10, according to the manufacturer's instructions. The gel was stained with Coomassie Brilliant Blue. The reduced forms in Panel A, lanes 1 to 9, were Serva pI maker proteins, ETT, ETT-Ser^{22}, ETT-Ser^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, ETT-Ala^{25}, and ETT-Ser^{78}-Ser^{82}, respectively. The oxidized forms in Panel B, lanes 1 to 10, were pI standards, ETT, ETT-Ser^{22}, ETT-Ser^{25}, ETT-Val^{26}, ETT-Gln^{27}, ETT-$Val^{26}Gln^{27}$, ETT-Ala^{25} (<0.1 ug), ETT-Ser^{78}-Ser^{82}, and ETT-Ala^{25}, respectively.


pI 1 2 3 4 5 6 7 8 9 10
3.50
4.40
5.34
5.90
6.90
7.30
7.70
8.30
9.45
10.65
B
pI 1 2 3 4 5 6 7 8 9
3.50
4.40
5.34
5.90
6.90
7.30
7.70
8.30
9.45
10.65
A

position 22 caused a pI change in both reduced (0.5 pH units) and oxidized (0.4 pH units) forms, however, the same substitutions at positions 25, 78 and 82 had no influence on the pI values (Fig. 18). These results provide additional evidence that Cys^{22} is the only cysteine residue in its thiolated form ($-S^-$) at a physiological pH, and that it is exposed or partially exposed to the molecular surface, whereas Cys^{25}, Cys^{78} and Cys^{82} are either in their sulfhydryl forms (-SH) or buried in the protein core. The three dimensional structures of E. coli (19) and T4 (20) thioredoxins have shown that their active sites are parts of a protrusions of the molecules. So it is possible that the active site of thioltransferase (glutaredoxin) is on the surface of the molecule, and only this location of the active center can explain how the low molecular weight enzyme interacts with its protein substrates (usually much larger than the enzyme in size), such as pyruvate kinase (1) and ribonucleotide reductase (21). Certainly, this proposal needs further support from the three dimensional structure of thioltransferase, and such studies are now in progress. There are two bands for oxidized forms of ETT-Ser^{25} and ETT-Ala^{25} (Panel B of Fig. 18). We think the upper one is the unoxidized (reduced) form and the lower one is the oxidized form for both mutants.

Striking pI shifts were observed in both reduced forms and oxidized forms when the two basic amino acid residues were replaced by neutral amino acids either individually or together. Compared with the wild-type enzyme (ETT), mutants of ETT-Val^{26} (Arg to Val), ETT-Gln^{27} (Lys to Gln) and ETT-$Val^{26}Gln^{27}$ (Arg-Lys to Val-Gln) have more acidic pIs, and the double mutant has the most acidic pI. These pI changes apparently resulted from the loss of the positively charged Arg and Lys

or both in the active center possibly located on the molecular surface. We showed in a previous paper that the enzyme activity of mutants of ETT-Val^{26}, ETT-Gln^{27} and ETT-Val^{26}Gln^{27} are only 32%, 67% and 5% of that of the wild-type (16). Thus the two basic amino acids, Arg^{26} and Lys^{27}, are very likely involved in the catalytic activity (see below).

Optimum pH and Kinetic Behavior-- Thiol-disulfide exchange activities of the wild-type and the mutant enzymes were assayed over the pH range of 5.5-9.5 using different amounts of protein. The general pattern of thioltransferase activity dependence on pH for each enzyme was similar with low rates at acidic pHs and with maximum rates at pH 8.5-9.0 (Fig. 19). Thus, modifications at the active center did not change the optimum pH of the enzyme, excluding the totally inactive mutant ETT-Ser^{22}. These results imply that the amino acid substitutions at the active center did not cause major conformational differences and the activity changes were the result of the active site alterations. Future three dimensional X-ray crystallographic analysis should establish the validity of this speculation.

The kinetic property of each thioltransferase with respect to the Cys-SO_3^- concentration is shown in Fig. 20. The v vs [S] plots of the wild-type and mutant thioltransferases showed non-Michaelis-Menten kinetics, i.e. at high substrate concentration, the enzyme activity was inhibited. The values of $K_{0.5}$ for these enzymes were estimated to be 0.5-0.8 mM. Our unpublished data clearly showed substrate (Cys-SO_3^- or cystine) inhibition of the thioltransferases, and the products of thioltransferase action, cysteine and HSO_3^-, were inhibitors of glutathione reductase. But the latter inhibition could be neglected in the current

Fig. 19. Optimum pH of mutant Thioltransferases. TT activity dependence on pH was compared among the mutants and wild-type proteins. The activities of wild-type and mutant thioltransferases were measured by the standard assay system in which 137 mM sodium phosphate (from pH 5.5 to 7.5) and 137 mM Tris-Hcl (from pH 8.0 to 9.5) were used. In each assay, 0.25 μg ETT, 0.20 μg ETT-Ser^{25}, 1.0 μg ETT-Ala^{25}, 0.90 μg ETT-Val^{26}, 0.4 μg ETT-Gln^{27}, 3.0 μg ETT-$Val^{26}Gln^{27}$, and 0.27 μg ETT-Ser^{78}-Ser^{82} was used, separately. Each value is the average of three separate experiments.

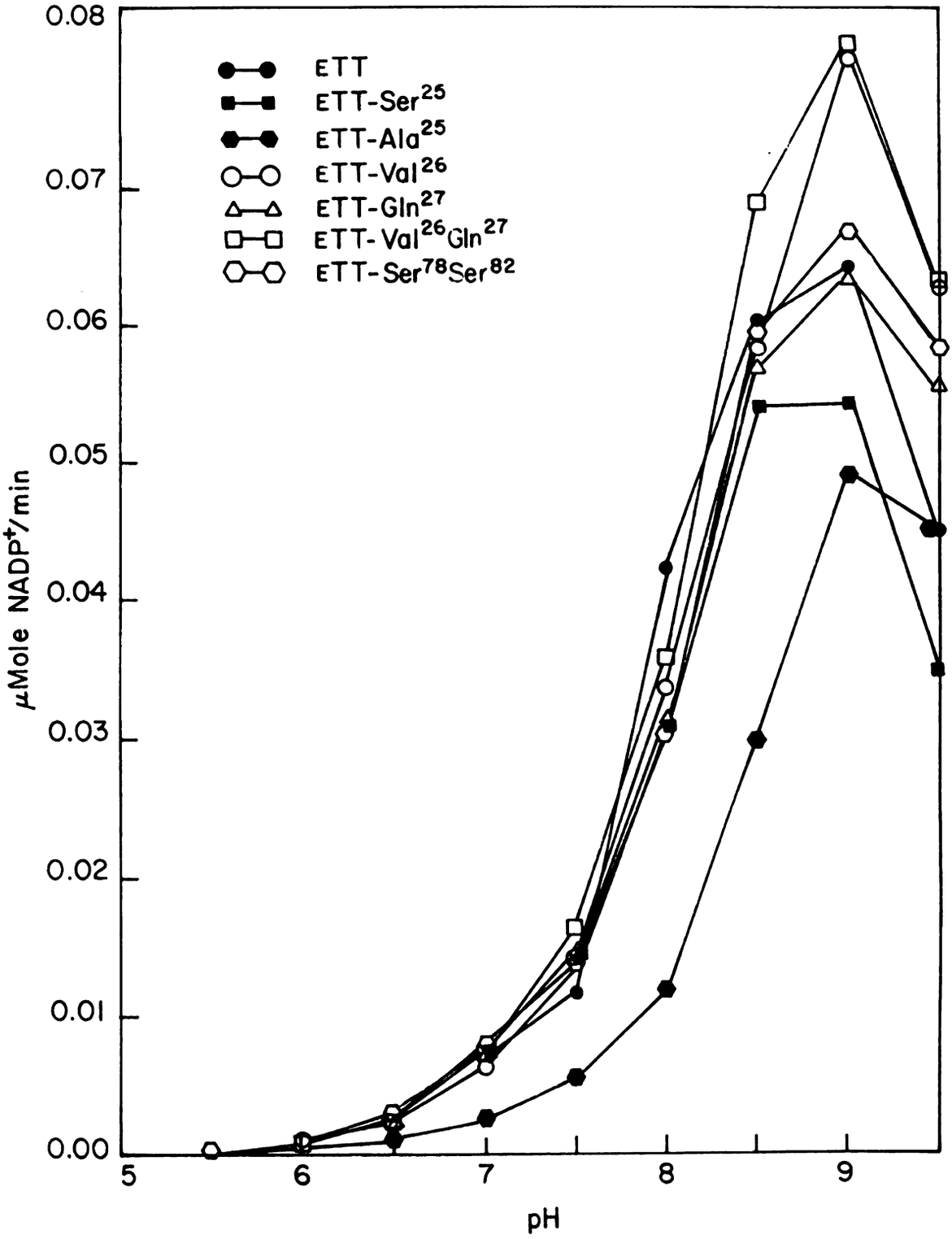
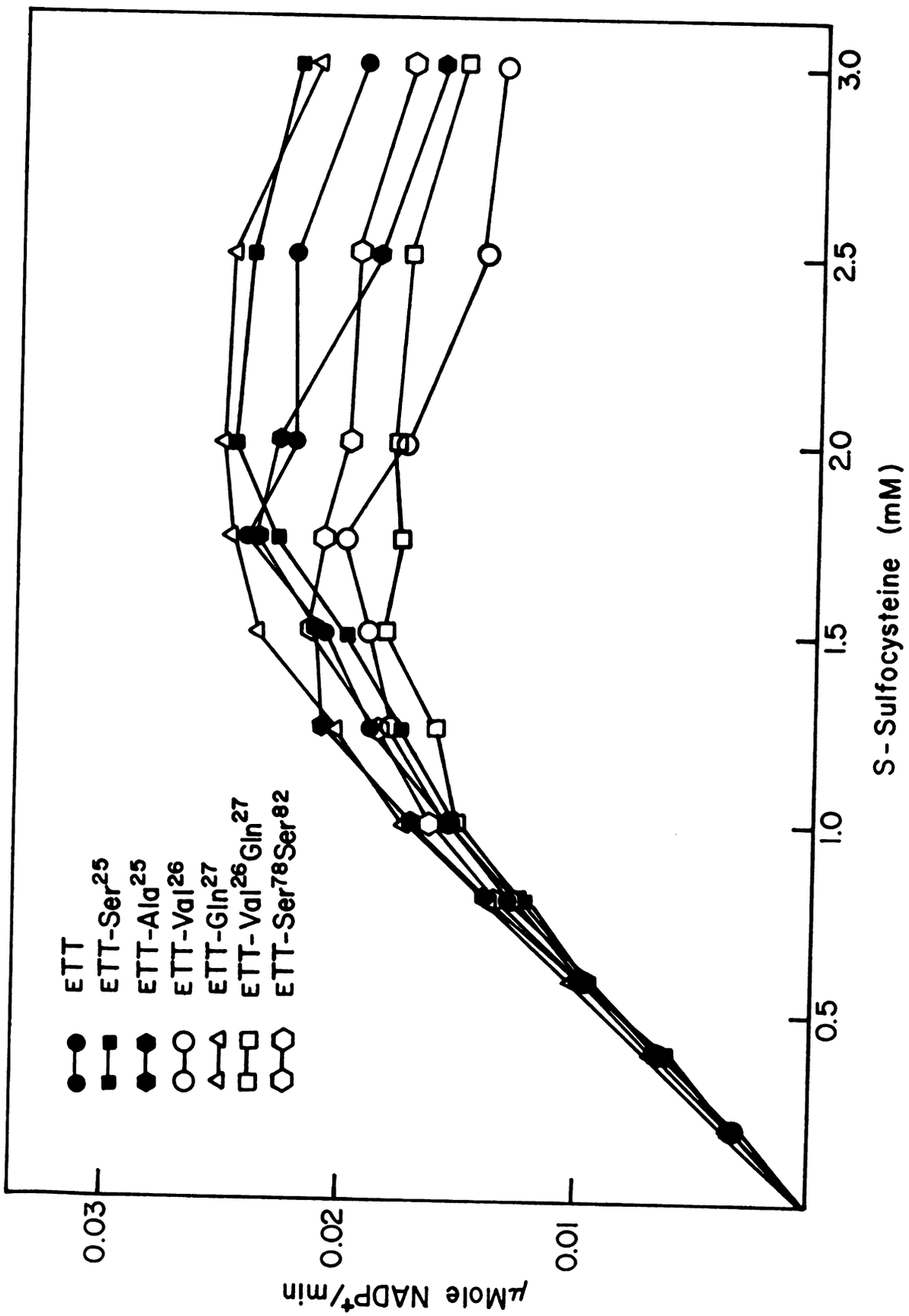

ETT
ETT-Ser25
ETT-Ala25
ETT-Val26
ETT-Gln27
ETT-Val26Gln27
ETT-Ser78Ser82
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
µMole NADP+/min
5
6
7
8
9
pH

Fig. 20. Kinetic behavior of mutant Thioltransferases.
Thioltransferase activity dependence on S-sulfocysteine concentration was compared for the wild-type and mutant enzymes. The standard assay (see Experimental Procedures) was used with increasing S-sulfocysteine concentrations. In each separate assay, 0.35 μg ETT, 0.35 μg ETT-Ser^{25}, 3.0 μg ETT-Ala^{25}, 0.85 μg ETT-Val^{26}, 0.65. μg ETT-Gln^{27}, 4.5 μg ETT-$Val^{26}Gln^{27}$, and 0.36 μg ETT-Ser^{78}-Ser^{82} were used, Each value is the average of three different experiments.


ETT
ETT-Ser25
ETT-Ala25
ETT-Val26
ETT-Gln27
ETT-Val26Gln27
ETT-Ser78Ser82
0.03
0.02
0.01
μMole NADP+/min
0.5
1.0
1.5
2.0
2.5
3.0
S-Sulfocysteine (mM)

study because of the excess of glutathione reductase and the negligible concentration of cysteine and HSO_3^- at the initial stages of the reactions.

The pK_a Values of the Expressed Thioltransferases--

Thioltransferase activity can be irreversibly inhibited by iodoacetic acid and iodoacetamide by alkylation of its active site-Cys^{11}, and the oxidized (intramolecular disulfide or mixed disulfide) form of the enzyme is protected against the alkylating reagents (12). It is known that the alkylation of thiols only occurs on their thiolate ($-S^-$) forms, and this reaction is, therefore, a strongly pH dependent process (13, 22). Thus the enzyme activity measured during an alkylation reaction must be a measure of unalkylated reduced (free) thioltransferase. If the same initial concentration of reduced thioltransferase and iodoacetamide are incubated together, this carboxamidomethylation reaction should obey the kinetics of a second order reaction as shown:

$$kt = [TT\text{-}AL] / [TT\text{-}SH] ([TT\text{-}SH] - [TT\text{-}AL])$$

$$\text{or } kt = 1 / ([TT\text{-}SH] - [TT\text{-}AL]) - 1 / [TT\text{-}SH]$$

where k is the apparent rate constant, t is the time, [TT-SH] represents the initial concentration of reduced thioltransferase, and [TT-AL] represents the concentration of the alkylated enzyme. As indicated above, the item [TT-SH] - [TT-AL] reflects the amount of reduced enzyme which is the function of the thioltransferase activity measured at time t.

The reduced wild-type and mutant thioltransferases (60 μM) were incubated with the same concentration of iodoacetamide (60 μM), separately, in the presence of 100 mM sodium citrate buffer (pH 2.5-5.5) or sodium phosphate buffer (pH 6.0-9.0) at room temperature. At the incubation times of 0, 5, 15, 25, 35, and 45 min, samples were withdrawn

and the enzyme activities were measured. At each specific pH, a plot of 1/([TT-SH] - [TT-AL]) (i.e., 1/thioltransferase activity) against time, t, gave a straight line and the slope of the straight line gave an apparent rate constant, k_{app}, of the alkylation reaction at a specific pH. One example is shown in Fig. 21, in which 60 μM of mutant ETT-Ser^{25} and 60 μM of iodoacetamide were incubated in 100 mM sodium phosphate buffer, pH 6.8 at room temperature, the plot of 1/enzyme activity vs t gave a straight line, the apparent rate constant, k_{app}, was 5.5 $mM^{-1}min^{-1}$ and the corresponding half-time, $t_{1/2}$, of the reaction was 3.0 min. Similar plots at various designated pHs for each enzyme were drawn (data not shown) and their k_{app} values were calculated. The pK_a values of the Cys^{22} sulfhydryl group for each enzyme was obtained from the midpoint of plots of k_{app} vs pH (Fig. 22). For the expressed wild-type thioltransferase (ETT), the apparent rate constant was pH dependent over the pH region of 3.0 to 4.5, whereas it was pH independent between pH 4.5-8.5. Since alkylation of thiols only occurs in the thiolate form ($-S^-$) (13,22), the extremely low k_{app} values below pH 3 indicated that Cys^{22} was in the sulfhydryl form (-SH) and not sensitive to the alyklating reagent. The increasing k_{app} values between pH 3.0-4.5 signified that the deprotonation of Cys^{22} sulfhydryl occurred in this pH range and the thiolate forms reacted with iodoacetamide. The unchanged k_{app} values over the pH region of 4.5 to 8.5 implied that the maximum rate of alkylation reaction was reached and all Cys^{22} side chains were in the thiolate form. The pK_a value of Cys^{22} of the wild-type recombinant thioltransferase was about 3.8, consistent with that of the native enzyme (12). Thus, the acetylation at the N-terminus of the native pig liver enzyme has no influence on the pK_a of Cys^{22}. For the mutant enzymes,

Fig. 21. A plot of the alkylation reaction between the same concentrations (60 μM) of reduced ETT-Ser^{25} and iodoacetamide. This reaction was performed at room temperature in 100 mM sodium phosphate buffer, pH 6.8. The values of reduced thioltransferase at various times were determined by enzyme activity assay after dilution (see text for details). The second order apparent rate constant, k_{app}, of the reaction was 5.5 $mM^{-1}min^{-1}$ and the half-time was 3.0 min.

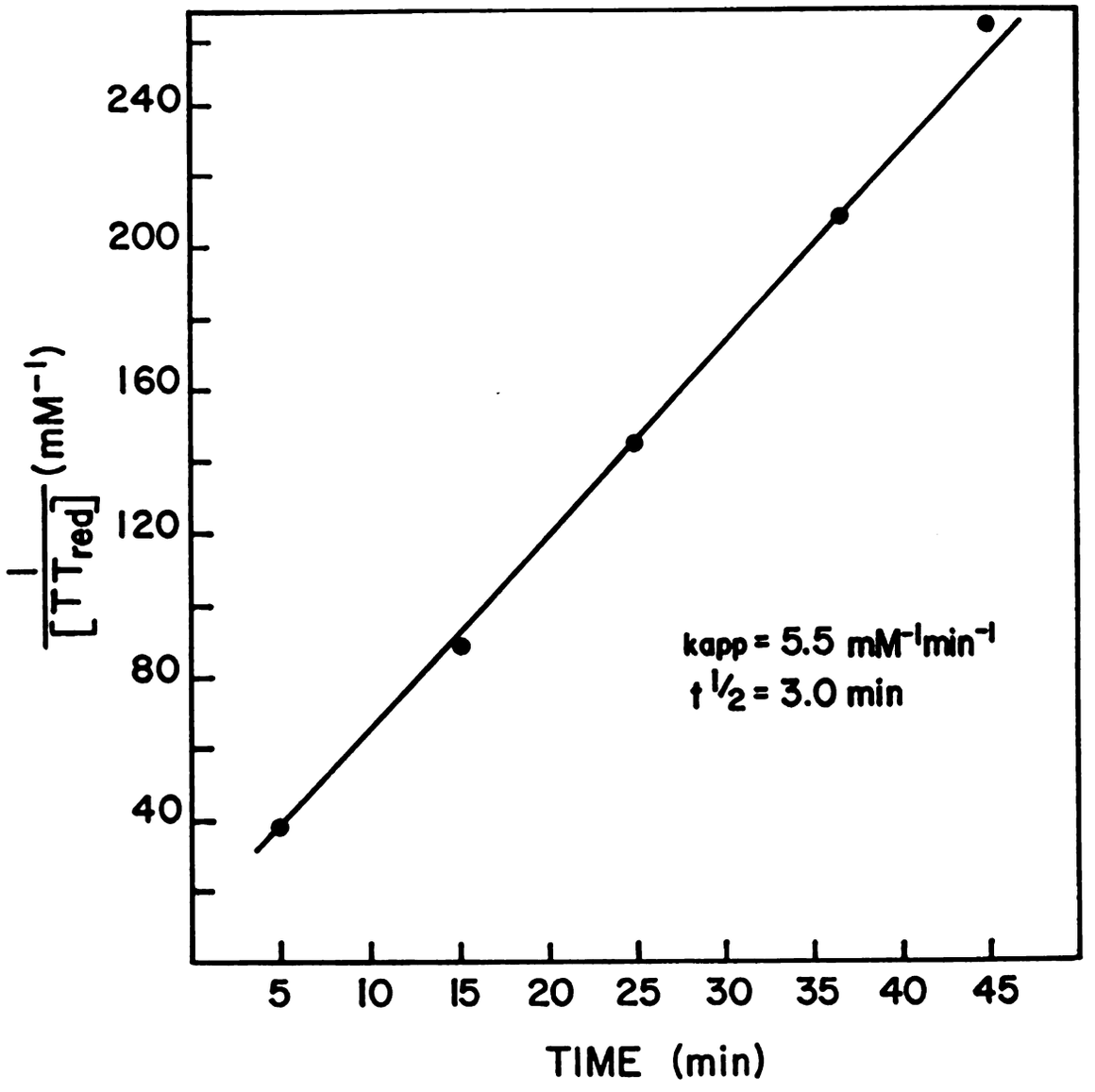
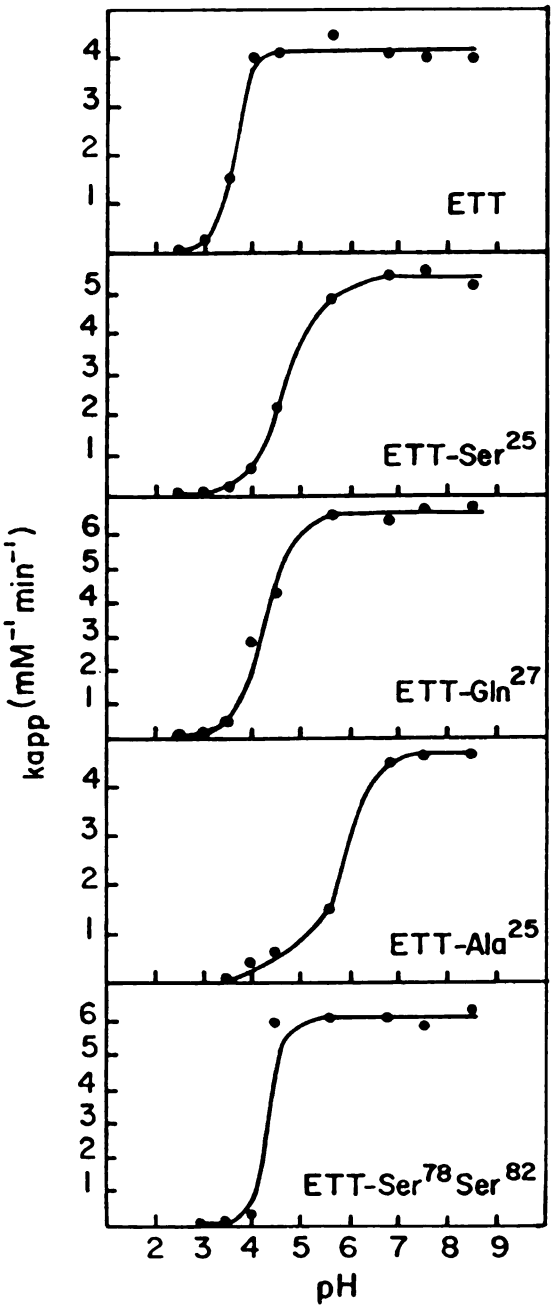

240
200
160
120
80
40
$\frac{1}{[TT_{red}]}$ (mM^{-1})
kapp = 5.5 $mM^{-1}min^{-1}$
$t^{1/2}$ = 3.0 min
5
10
15
20
25
30
35
40
45
TIME (min)

Fig. 22. pH dependence of second order apparent rate constants of the reactions between each of the reduced thioltransferases (60 μM) and iodoacetamide (60 μM). The k_{app} values of the reactions were obtained as described in Fig. 4 and Experimental Procedures.


ETT
ETT-Ser25
ETT-Gln27
ETT-Ala25
ETT-Ser78Ser82
kapp (mM-1 min-1)
pH
2 3 4 5 6 7 8 9

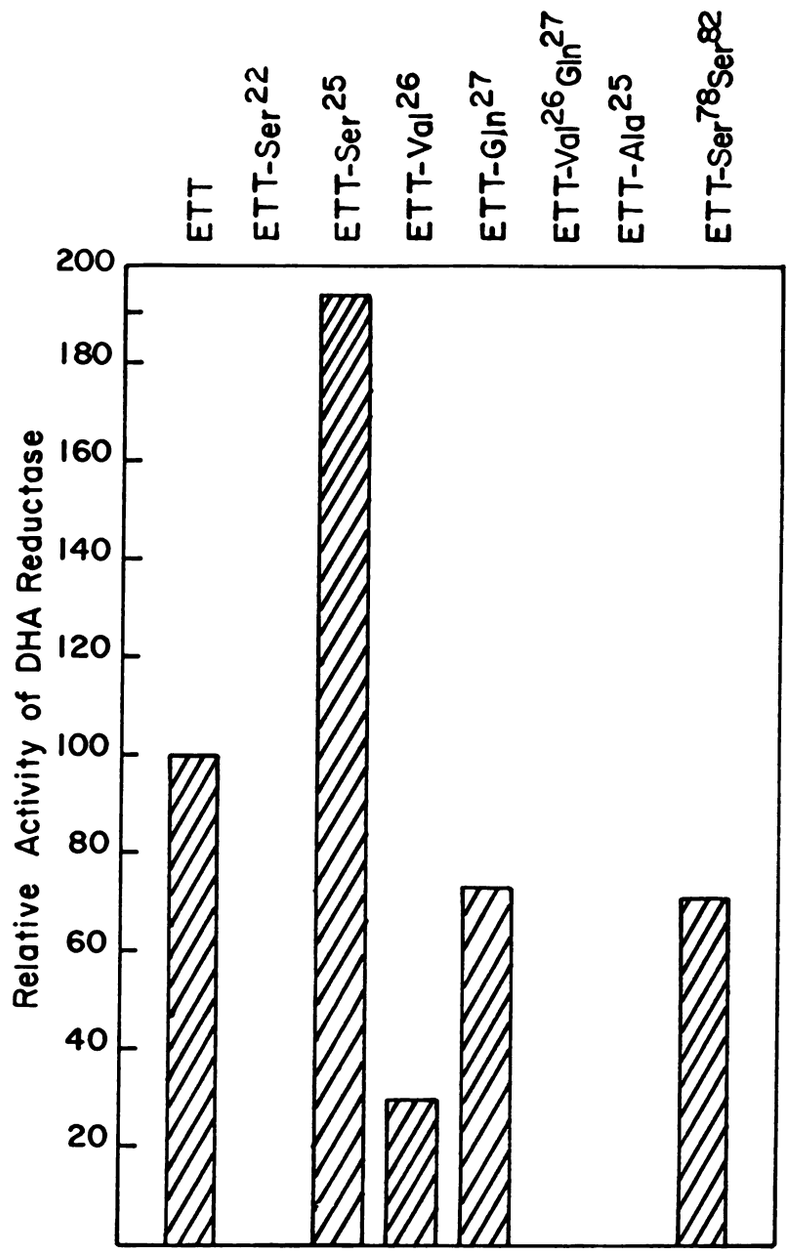
ETT-Ser^{25}, ETT-Gln^{27}, ETT-Ala^{25}, and ETT-Ser^{78}-Ser^{82}, the pH sensitive k_{app} values were in the regions of 4.0 to 6.0, 3.5 to 5.5, 4.5 to 6.5, and 4.0 to 5.5, respectively. The pK_a values of the Cys^{22} side chain were estimated to be 4.9 for ETT-Ser^{25}, 4.3 for ETT-Gln^{27}, 5.9 for ETT-Ala^{25}, and 4.4 for ETT-Ser^{78}-Ser^{82}, all more basic than that of the wild-type. Substitution of the cysteine with either a serine or an alanine at position 25 caused a more basic shift of the pK_a of Cys^{22} than the changes at position 27 (Lys^{27} to Gln^{27}) and position 78 and 82 (Cys^{78}-Cys^{82} to Ser^{78}-Ser^{82}). Lys^{27} as well as Arg^{26} was speculated to facilitate the low pK_a at Cys^{22}, but a change of Lys^{27} to Gln^{27} only slightly increased the pK_a of Cys^{22} (ca. 0.5 pH units) and decreased the activity by 33 % (16), whereas a greater pK_a increase was caused by the change of Cys^{25} to Ser^{25}, yet enzyme activity was raised 10% (16). Thus, for mutant ETT-Gln^{27}, the 33% loss of enzyme activity was not the result of the slight pK_a increase, but Lys^{27} likely played some other role in the enzyme catalytic mechanism. Currently, we do not know the function of Lys^{27}, although one possibility is that this residue can stabilize the enzyme-substrate intermediate by ionic interactions between its positively charged side chain and a negatively charged group of the substrates, e.g., GSH. It is interesting that replacing Cys^{25} with Ser^{25} and with Ala^{25}, separately, resulted in different pK_a changes (1.1 and 2.1 pH units, respectively) at Cys^{22} and resulted in totally different activity alterations (10% increase vs 91% decrease, respectively) (16). Compared with serine, the relatively more hydrophobic alanine replacing Cys at this position might disturb the local three dimensional structure of the active center and cause Cys^{22} to be less exposed. The exchanges of the two downstream cysteines, Cys^{78} and Cys^{82}, with two serines had little influence on the pK_a value of Cys^{22}.

group is not facilitated by either Cys^{25}, Lys^{27}, or Cys^{18} and Cys^{22}.

In contrast, the amino acid responsible for the low pK_a at Cys^{22} was Arg^{26}. We could not measure the pK_a value of mutants ETT-Val^{26} and ETT-$Val^{26}Gln^{27}$, because the apparent rate constants, k_{app}, of both mutants were very low (about 0.4 $mM^{-1}min^{-1}$) and pH independent over the region of 2.5 to 8.5., that is, the two mutants were not sensitive to iodoacetamide. These results clearly indicated that replacing Arg^{26} with Val^{26} significantly decreased the deprotonation of the active site sulfhydryl group. We conclude that the role of Arg^{26} is to facilitate the low pK_a of Cys^{22}, i.e., enhance its S^- nucleophilicity, necessary for the thioltransferase catalytic reaction.

The DHA Reductase Activity-- In the presence of GSH, thioltransferase can catalyze the reduction of DHA to ascorbic acid (15). The intrinsic DHA reductase activity of the wild-type and each of the mutant enzymes was measured as described in Experimental Procedures and compared with each other based on the same amount of protein (0.4 μg) (Fig. 23). With the activity of the wild-type enzyme defined as 100%, the relative activities of the mutants were 0% for ETT-Ser^{22}, ETT-Val^{26}-Gln^{27}, and ETT-Ala^{25}, 194% for ETT-Ser^{25}, 30% for ETT-Val^{26}, 73% for ETT-Gln^{27}, and 71% for ETT-Ser^{18}-Ser^{22}. Like the thiol-disulfide exchange activity, the Ser^{22} mutant had no detectable DHA reductase activity. This result indicates that Cys^{22} is very likely the catalytic site for both intrinsic activities. Compared with the thiol-disulfide exchange activity, ETT-Ser^{25} had a significantly greater DHA reductase activity, whereas there was no detectable activity for ETT-Ala^{25}. The evidence suggests that a serine is more favored than a cysteine at position 25, especially for DHA reductase activity. The DHA reductase activity of

Fig. 23. Comparison of the DHA reductase activity of thioltransferases--The DHA reductase activities of the wild-type and mutant thioltransferases were measured based on the same amount of proteins (0.4 μg) as described in Experimental Procedures. The activity of the wild-type enzyme was defined as 100%, and the relative activities of mutant enzymes were compared.


ETT
ETT-Ser22
ETT-Ser25
ETT-Val26
ETT-Gln27
ETT-Val26Gln27
ETT-Ala25
ETT-Ser78Ser82
Relative Activity of DHA Reductase
200
180
160
140
120
100
80
60
40
20

for DHA reductase activity. The DHA reductase activity of thioltransferase is less active in mutants of Val^{26}, Gln^{27}, Ser^{18}-Ser^{82}, and Val^{26}-Gln^{27}, than the corresponding thiol-disulfide exchange activity. The co-existence of the two basic amino acids, Arg^{26} and Lys^{27}, may be essential for both intrinsic activities of thioltransferase.

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CHAPTER VI

EVIDENCES FOR THE CATALYTIC MECHANISM OF THIOLTRANSFERASE

SUMMARY

Two thioltransferase catalytic mechanisms have been proposed (Askelöf, et al., (1974) FEBS Lett. 38, 263-267, and Gan, and Wells (1987) J. Biol. Chem. 262, 6704-6707). To test these proposals, seven mutant pig liver thioltransferases, created by site-directed mutagenesis, were used in the present study. All the expressed enzymes, including wild-type and mutants with the exception of ETT-Ser^{22}, were variably inactivated by iodoacetamide, and similar results were obtained when these enzymes were preincubated with GSH. However, when preincubated with S-sulfocysteine or hydroxyethyl disulfide, the activity of the enzymes was totally or partially protected against inhibition by iodoacetamide, with the exception of the mutants, ETT-Ser^{25} and ETT-Ala^{25}. However, when simultaneously pretreated with GSH and S-sulfocysteine, all enzymes were protected. Isoelectric focusing analysis of the above preincubation mixtures showed that different enzyme-substrate intermediates occurred. Using radioactively labeled substrates, [U-^{14}C]-cystine and [glycine-2-^{3}H]GSH, enzyme-substrate intermediates were also detected. The data indicate that reduced thioltransferase reacts first with disulfide substrates then with substrate GSH, and the formation of either mixed disulfide or intramolecular disulfide protected the enzyme from inactivation by iodoacetamide. Based on experimental results, alternative models of the catalytic mechanism of wild-type and mutant thioltransferases are proposed.

INTRODUCTION

Thioltransferase, also called glutaredoxin, has been known for more than 35 years (1). This low molecular weight, heat stable cytosolic enzyme is widely distributed in bacteria, yeast, and animals, and its thiol-disulfide exchange catalytic properties and primary structure have been studied (2-4). Coupled with glutathione reductase and NADPH, thioltransferase catalyzes reversible thiol-disulfide exchange reactions in the presence of GSH.

$$RSSR + 2\ GSH \xrightleftharpoons{\text{thioltransferase}} GSSG + 2\ RSH \qquad (1)$$

$$GSSG + NADPH + H^{+} \xrightleftharpoons{\text{glutathione reductase}} 2\ GSH + NADP^{+} \qquad (2)$$

the net reaction is:

$$RSSR + NADPH + H^{+} \rightleftharpoons 2\ RSH + NADP^{+} \qquad (3)$$

where RSSR is any suitable disulfide substrate, including proteins. The whole reaction system may play an important role in the regulation of enzyme activity and/or in maintaining a normal cellular thiol/disulfide ratio (5, 6). Askelöf, et al. (7) proposed that the thioltransferase catalyzed reaction (Reaction 1) was a consecutive two step ionic displacement reaction.

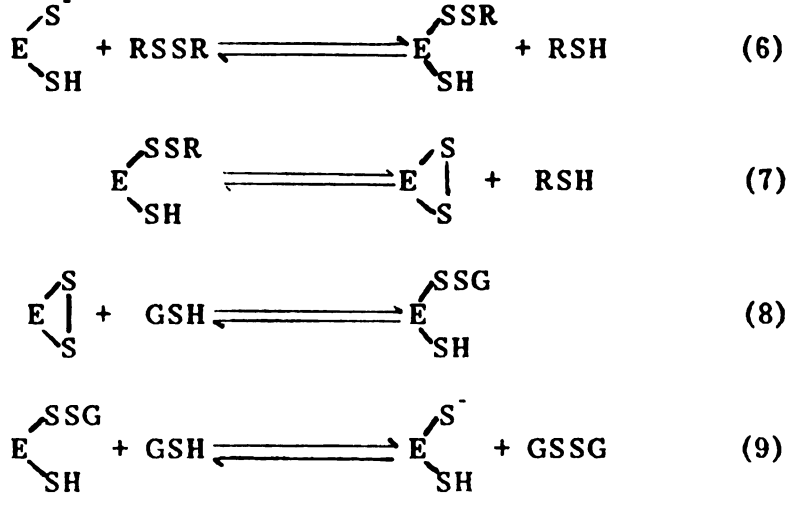
$$RSSR + GSH \rightleftharpoons RSSG + RSH \qquad (4)$$

$$RSSG + GSH \rightleftharpoons GSSG + RSH \qquad (5)$$

in which there is expected formation of a mixed disulfide intermediate (RSSG) between the half-disulfide substrate e.g., $ArSSAr^{1}$ and GSH. However, no evidence for a mixed disulfide intermediate was found when

cystine was the disulfide substrate. Reactions 4 and 5 do not explain how the enzyme may participate in the catalytic process.

Alkylating reagents, iodoacetic acid (IAA) and iodoacetamide (IAM), can irreversibly inactivate thioltransferase by alkylation of the enzyme at the sulfhydryl group of its active site, Cys^{22}, in a pH dependent manner (8). Since the alkylation of thiols only occurs in the deprotonated thiolate forms (9, 10), the formation of a mixed disulfide or an intramolecular disulfide at the active site of thioltransferase should make the enzyme insensitive to alkylating reagents. During identification of the active site of pig liver thioltransferase by inhibition studies, Gan and Wells found that preincubation of the enzyme with cystine could protect the enzyme from inactivation by iodoacetic acid, and suggested a model for the thioltransferase catalytic mechanism as follows:



where E, $-S^-$, and -SH represented the enzyme, Cys^{22}, and Cys^{25}, respectively (8).

In Chapters IV and V (11, 12), we described the creation of seven

mutant pig liver thioltransferases by site-directed mutagenesis, directly identified the essential amino acids at the active center, and characterized these mutants. The most surprising finding was that exchange of the cysteine with a serine at position 25 caused an increase rather than a decrease in both thiol-disulfide exchange activity and DHA reductase activity of the enzyme. The inability for the formation of a disulfide bond between Cys^{22} and Cys^{25} in the Ser^{25} mutant did not reduce the enzyme's catalytic efficiency, and this made us consider an alternative mechanism illustrated by Reactions 10-12 for this mutant.

In this article, taking advantage of these mutants, we critically investigated the catalytic reactions of thioltransferase by various inhibition studies, isoelectric focusing of enzyme-substrate complexes, and by reactions with radioactive labeled substrates. Our results support Reactions 6-9 for the native enzyme, and suggest a three-step mechanism, Reactions 10-12 for mutants of Cys^{25}.

EXPERIMENTAL PROCEDURES

Materials-- L-cystine, dithiothreitol (DTT), GSH, iodoacetamide and iodoacetic acid were from Sigma; 2-hydroxylethyl disulfide (HED) was from Aldrich Chemical Company, Inc.; L-[U-^{14}C]cystine with a specific activity of 300 mCi/mmol and [glycine-2-^{3}H]GSH with a specific activity of 1 Ci/mmol were purchased from New England Nuclear; Isoelectric focusing gel and pI marker proteins were from Serva; Safety-solve and 2,5-diphenyloxazole (PPO) were from Research Products International Corp.; dimethyl sulfoxide (DMSO) was obtained from J. T. Baker Inc.

Preincubation and Inhibition Studies of Mutant Thioltransferases-- The reduced wild-type and mutant pig liver thioltransferases were prepared as described previously (11,12). Each of the enzymes (0.06 mM) was incubated with 0.12 mM IAM in the presence of 100 mM sodium phosphate buffer, pH7.5, at room temperature. At various times, samples were withdrawn, and the enzyme activity was assayed using the standard system. The IAM inhibition experiments were also performed after preincubating each enzyme (0.06 mM) with GSH (0.5 mM), Cys-SO_3^- (2.5 mM), GSH (0.5 mM)/Cys-SO_3^- (2.5 mM), and HED (2.5 mM), separately, in the presence of 100 mM sodium phosphate buffer, pH 7.5, at room temperature for 15 min.

Isoelectric Focusing Analysis-- The wild-type thioltransferase (ETT), Ser^{25} mutant (ETT-Ser^{25}) and Ser^{78}-Ser^{82} mutant (ETT-Ser^{78}-Ser^{82}), 3 μg each, were treated with 2.5 mM HED, 2.5 mM cystine, 2.5 mM Cys-SO_3^- and 0.5 mM GSH/2.5 mM Cys-SO_3^-, separately, in 100 mM sodium phosphate buffer, pH 7.5, at room temperature for 15 min. Each of the incubation mixtures was then analyzed by isoelectric focusing as described previously (12).

Radioactive Labeling Studies-- Two radioactively labeled substrates, [^{14}C-]cystine and [^{3}H-]GSH were used for the purpose of tracking the formation of enzyme-substrate intermediates.

[^{14}C]cystine-- In a volume of 100 µl, each of the reduced wild-type and mutant thioltransferases (0.3 mM) were incubated with a mixture of [^{14}C]cystine (ca. 0.3 µCi) and 0.6 mM nonisotopic cystine in 100 mM sodium phosphate buffer, pH 7.5, at room temperature for 20 min. Excess labeled substrate was separated from the enzymes on a Sephadex G-25 column (1 x 45 cm) which was equilibrated with 20 mM sodium phosphate buffer, pH 7.5. The catalytically active fractions (or the A_{280} peak for mutant ETT-Ser^{22}) were collected and the total counts were measured by liquid scintillation spectrometry. Samples were then concentrated by Centriprep-10 concentrators (Amicon) following the manufacture's instructions, and the protein concentration and radioactity counts of the concentrated samples were determined.

[^{3}H]GSH-- In a volume of 100 µl, each reduced enzyme (0.3 mM) was incubated with a mixture of 12.5 mM Cys-SO_3^-, 1.0 µl of [^{3}H]GSH (Ca.1 µCi) and 2.5 mM nonisotopic GSH in the presence of 100 mM sodium phosphate buffer, pH 7.5 at room temperature for 20 min. Sephadex G-25 chromatography, concentration of the catalytically active fractions, protein concentration and radioactivity determinations were done as described above.

For control experiments, reduced wild-type enzyme (ETT) and the mutant, ETT-Ser^{25}, were incubated with [^{3}H]GSH in the absence of Cys-SO_3^- or ETT-Ser^{25} was pretreated with IAM before incubation with [^{14}C]cystine. The rest of the procedures were the same as mentioned above.

Nonreducing SDS-PAGE and Autoradiography-- The wild-type enzyme (ETT) and the mutant ETT-Ser^{25}, treated with [^{14}C]cystine or [^{3}H]GSH as described above, were analyzed by a 15% SDS- polyacrylamide gel under nonreducing condition (e.g., without DTT or ß-mercaptoethanol in the normal SDS-PAGE system). The gel was soaked in DMSO for 1 h with three changes, and then in a solution of 22.5% (w/v) PPO in DMSO for 1 h. After thoroughly rinsing with water, the gel was dried under vacuum and exposed to X-ray film (Kodak XAR-5) with an intensifying screen at -70°C for 2 days.

RESULTS

Preincubation and Inhibition Studies-- It is known that the alkylation of thiols only occurs in their thiolate forms (9, 10), and for pig liver thioltransferase, only the sulfhydryl group, Cys^{22}, the active site of the enzyme, is the target of the alkylating reagents at pH 7.5 (8, 11, 12). When incubated with IAM, more than 85% of the activities of the wild-type enzyme (ETT) and the mutants, ETT-Ser^{25}, ETT-Ala^{25}, ETT-Gln^{27}, and ETT-Ser^{18}-Ser^{82} were inhibited, whereas the mutants of ETT-Val^{26} and ETT-$Val^{26}Gln^{27}$ still had Ca. 70% and 90% activity remaining, respectively (Fig. 24). The latter two mutants were not sensitive to IAM inhibition at pH 7.5 since they had lost the ability to facilitate the deprotonation of their Cys^{22} side chain (12). The deactivation of other expressed enzymes by IAM was the result of their available thiolate side chain ($-S^-$) of Cys^{22}. These results were consistent with those of the previous pK_a measurement experiments (12).

Thioltransferase catalyzed thiol-disulfide interchange reaction (Reaction 1) involves the enzyme, disulfide substrate and GSH. The pseudodisulfide substrate, i.e., thiosulfate ester, used typically in our laboratory is Cys-SO_3^-. In order to test the possible mechanism, each of the wild-type and mutant thioltransferases, except ETT-Ser^{22}, was pretreated with either GSH, Cys-SO_3^- or GSH/Cys-SO_3^-, separately, and then incubated with IAM (Fig. 25). Preincubation of these enzymes with GSH led to the same results as those incubated directly with IAM. The only difference was that the two Val^{26} containing mutants were relatively less sensitive to IAM (Fig. 25, left). These results implied that GSH could not directly bind to the Cys^{22}, but might have some influence on it, since GSH slightly promoted the alkylation reaction presumably by

Fig. 24 Inhibition of pig liver thioltransferases. The wild-type and mutant thioltransferases (0.06 mM) were incubated for various times with iodoacetamide (0.12 mM) in 100 mM sodium phosphate buffer, pH 7.5 at room temperature, and the thiol-disulfide exchange activity of each enzyme was measured. The activity measured at zero time for each enzyme was defined as 100%, and the relative activities as a function of time were compared. The symbol for each enzyme is designated in the figure. Each value is the average of two separate experiments.

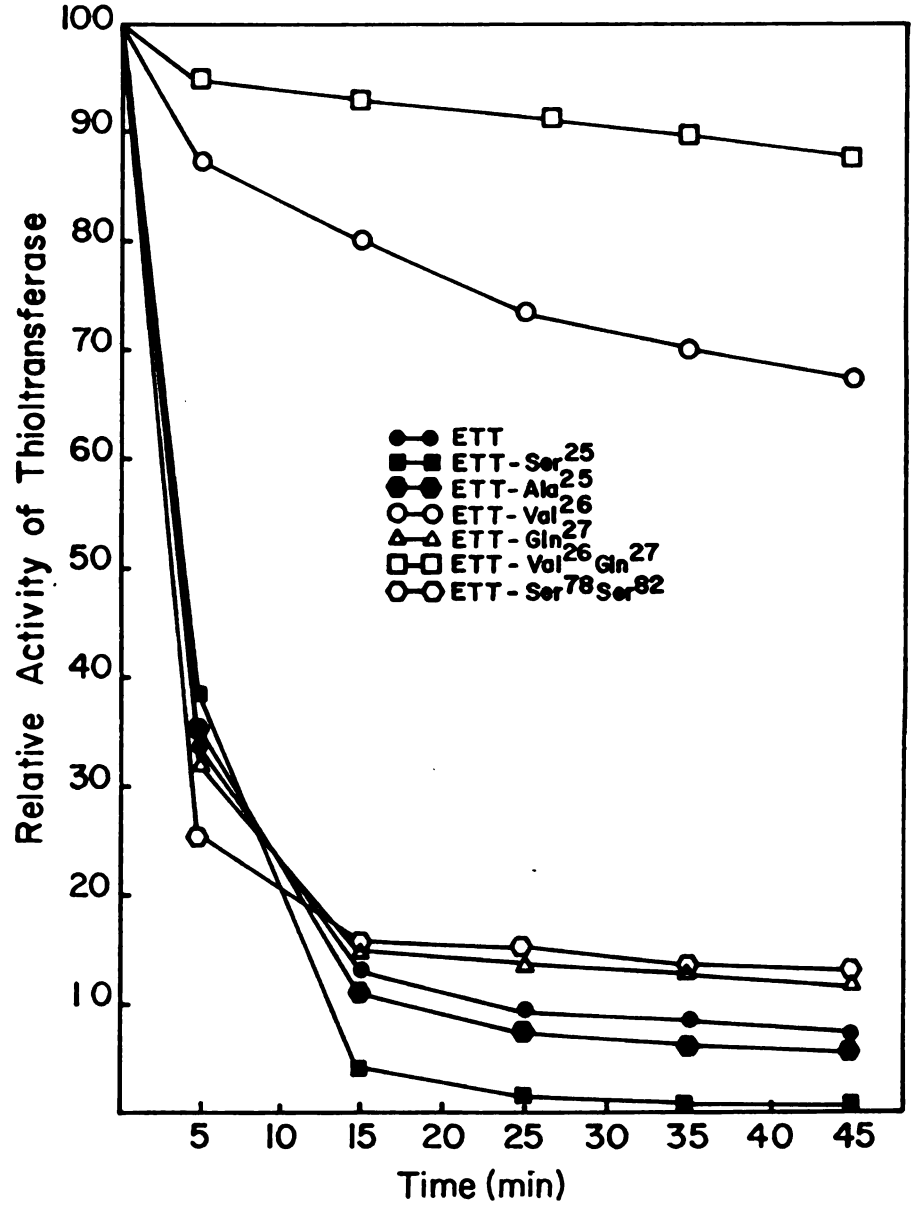
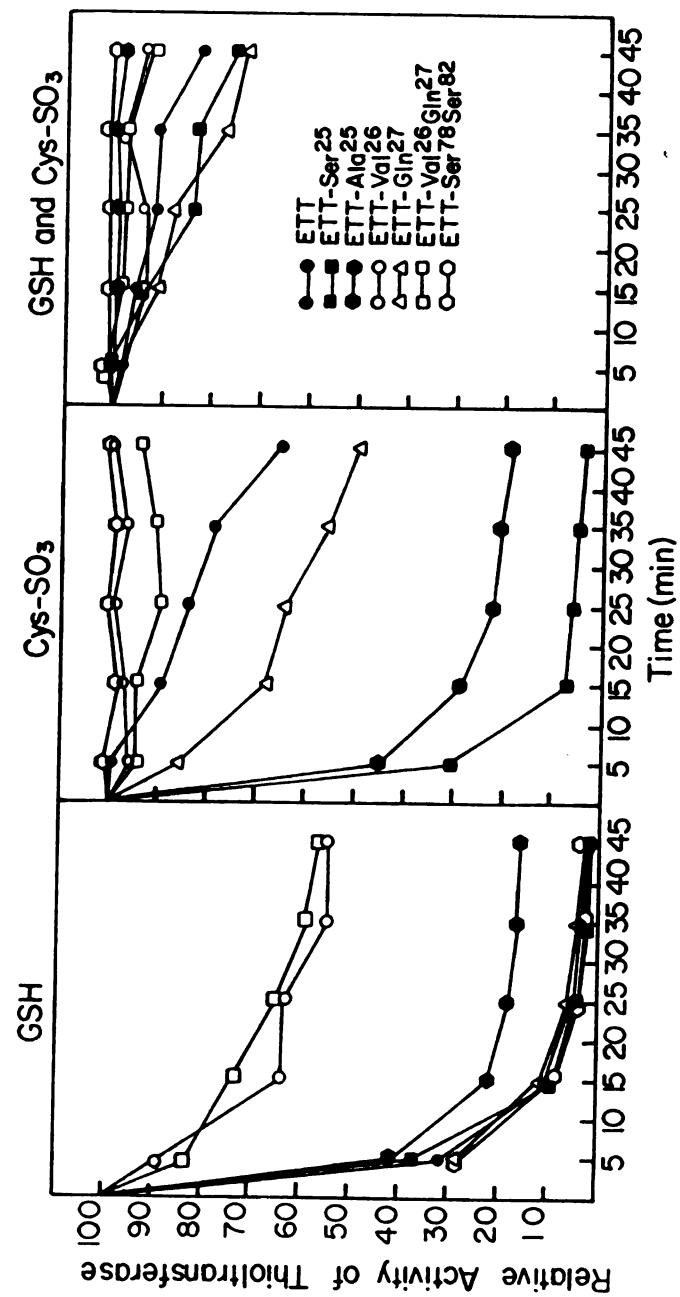

Relative Activity of Thioltransferase
100
90
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
Time (min)
ETT
ETT-Ser25
ETT-Ala25
ETT-Val26
ETT-Gln27
ETT-Val26Gln27
ETT-Ser78Ser82

Fig. 25 Differential preincubation of pig liver thioltransferases. Each of the wild-type and mutant thioltransferases (0.06 mM) was preincubated with 0.5 mM GSH (left), 2.5 mM Cys-SO_3^- (middle), and Cys-SO_3^- (2.5 mM)/GSH (0.5 mM) (right), separately, in the presence of sodium phosphate buffer, pH 7.5 at room temperature for 15 min, then 0.12 mM IAM were added. For the remaining steps, see the legend to Fig. 24. Each value is the average of two separate experiments.


GSH
Cys-SO3
GSH and Cys-SO3
Relative Activity of Thioltransferase
Time (min)
ETT
ETT-Ser25
ETT-Ala25
ETT-Val26
ETT-Gln27
ETT-Val26Gln27
ETT-Ser78Ser82

reducing previously air-oxidized forms of the enzymes. When pretreated with the disulfide-like substrate, Cys-SO_3^-, all enzymes were totally or partially protected from inactivation by IAM except the two Cys^{25} substituted mutants, ETT-Ser^{25} and ETT-Ala^{25} (Fig. 25, middle). Identical results were obtained using L-cystine instead of Cys-SO_3 (data not shown). ETT-Val^{26}, ETT-$Val^{26}Gln^{27}$ and ETT-Ser^{78}-Ser^{82} totally retained their activity by pretreatment with Cys-SO_3^-, possibly by the formation of an intramolecular disulfide (Reaction 6 and 7) which prevented IAM reaction with Cys^{22}. ETT-Ser^{25} and ETT-Ala^{25}, lacking the ability to establish an intramolecular disulfide bond within their active center, were inhibited by IAM even after the initial formation of a mixed disulfide bond between the enzyme and the substrate (Reaction 10), and in the presence of an excess of Cys-SO_3^- (Cys-SO_3^- : IAM : enzyme = 2.5 mM : 0.12 mM : 0.06 mM).

$$E\begin{matrix}S^- \\ SH(OH)\end{matrix} + RSSR \rightleftharpoons E\begin{matrix}SSR \\ SH(OH)\end{matrix} + RSH \qquad (10)$$

$$E\begin{matrix}S\text{-}SR \\ SH(OH)\end{matrix} + GSH \rightleftharpoons E\begin{matrix}S\text{-}SG \\ SH(OH)\end{matrix} + RSH \qquad (11)$$

$$E\begin{matrix}S\text{-}SG \\ SH(OH)\end{matrix} + GSH \rightleftharpoons E\begin{matrix}S^- \\ SH(OH)\end{matrix} + GSSG \qquad (12)$$

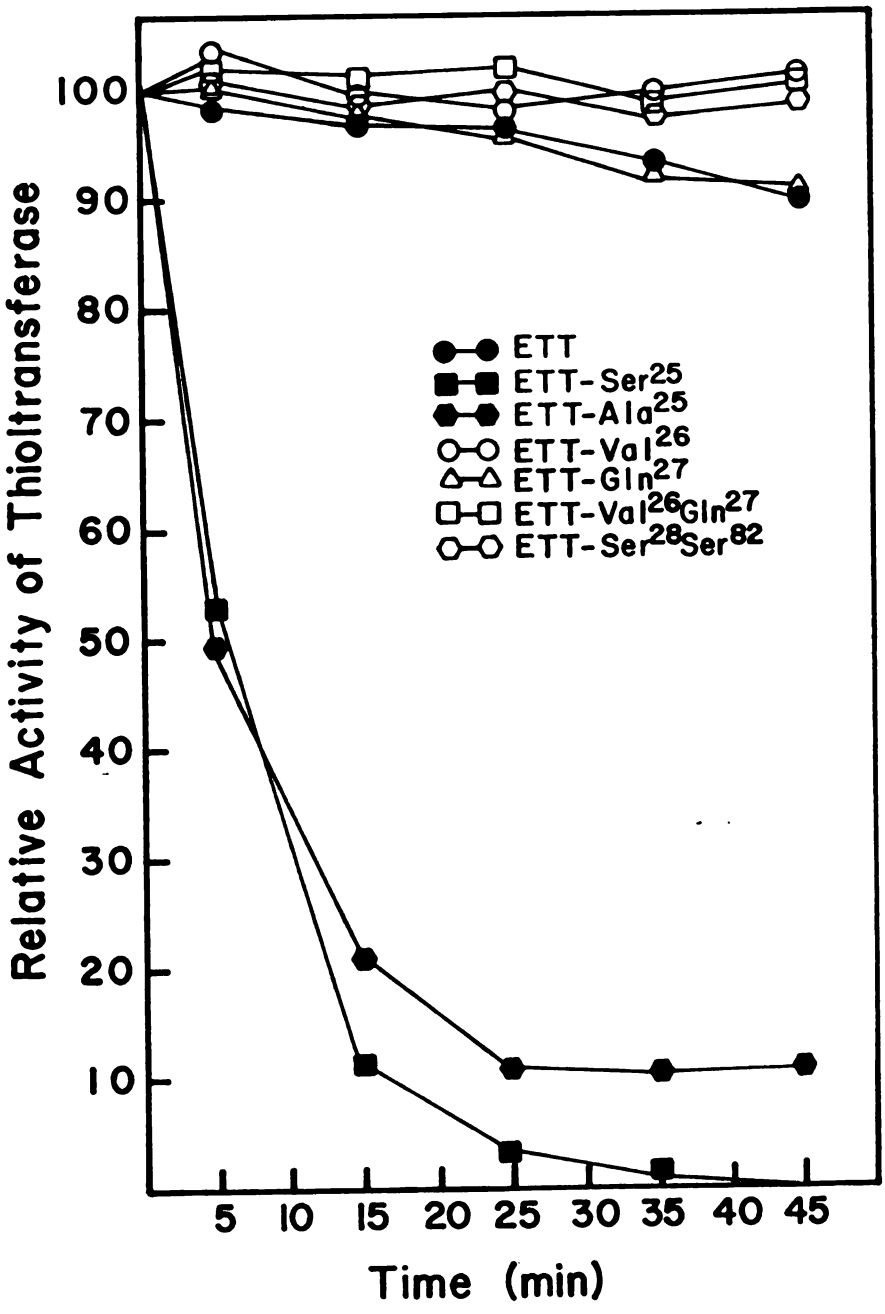
where (OH) represents the hydroxyl group of Ser^{25} of mutant ETT-Ser^{25}.

This phenomenon can be explained on the basis that Reaction 10 is reversible. IAM could remove the enzyme from the reaction by reacting with free enzyme and forming a dead end complex, i.e., pull the reaction to the left. For ETT and ETT-Gln^{27}, the mixed disulfide formed in Reaction 6 was competitively replaced either by the -SH group of Cys^{25}

to form an intramolecular disulfide (Reaction 7) or by the products to reverse the direction of Reaction 6. In this situation, the rate of the reversal of Reaction 6 was much slower, i.e., less free enzyme was available to IAM, and these two enzymes were only partially inactivated by IAM. It is of interest that when simultaneously preincubated with Cys-SO_3^- and GSH, all thioltransferases were relatively well protected, including the two mutants lacking Cys^{25} (Fig. 25, right). We believe that the coexistence of both substrates made the enzyme accessible to GSH to form an enzyme-SG mixed disulfide at Cys^{22} by replacing Cys^{25} (Reaction 8 or 11) or the mixed disulfide between enzyme and the initial disulfide substrate (Reaction 6 or 10). This newly formed disulfide bond may be relatively more stable than the enzyme-half-substrate disulfide bond and the final step of the process, Reaction 9 or 13, might be the rate determining step. It is likely that during the preincubation, the enzyme catalyzed reaction had reached a steady-state balance in which the enzyme was virtually saturated by the substrates, forming predominantly mixed and intramolecular disulfides, so that when IAM was added, little free enzyme was available. However, IAM gradually pulled some of the enzyme molecules from the system at a slow rate since partial inhibition of the activity was observed.

The enzymes were also pretreated with HED, and, except for the two Cys^{25} substituted mutants, all others were fully protected against inactivation by IAM (Fig. 26). Because of the two exceptions, this protection appears to be acquired by the formation of intramolecular disulfides. Similar studies were done using the native pig liver thioltransferase, this enzyme was fully protected when preincubated with cystine, Cys-SO_3^-, or HED, but not with GSH (data not shown) in

Fig. 26 Pig liver thioltransferases protection by HED. The protection experiments were performed by pretreating each of the wild-type and mutant thioltransferases (0.06 mM) with 2.5 mM HED before the addition of 0.12 mM IAM. The test conditions were the same as described in the legend of Fig. 25. Each value is the average of two separate experiments.


Relative Activity of Thioltransferase
100
90
80
70
60
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40
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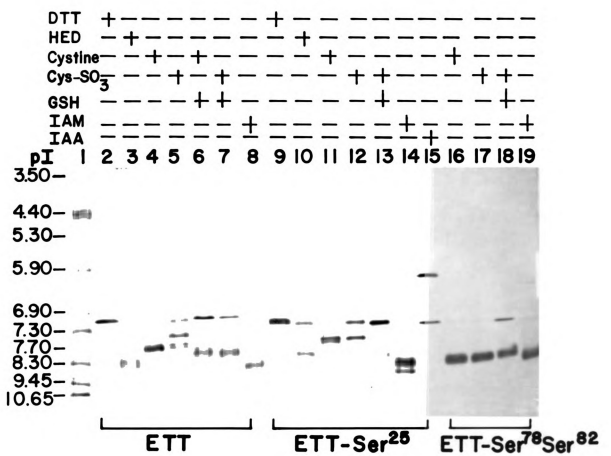
35
40
45
Time (min)
ETT
ETT-Ser25
ETT-Ala25
ETT-Val26
ETT-Gln27
ETT-Val26Gln27
ETT-Ser28Ser82

agreement with Gan and Wells (15). The only difference between the native and the recombinant enzyme is that the former has a N-acetylated N-terminus (13). The different sensitivity to IAM of the two enzymes when pretreated with cystine or Cys-SO_3^- implies teleologically that one possible role of acetylation at the N-terminus is protection of the native enzyme against physiological alkylation inactivation.

Isoelectric Focusing Analysis-- The inhibition studies described above could not reveal what kinds of enzyme-substrate intermediates were formed. We have already demonstrated that the exchange of Cys^{22} with Ser caused a protein pI shift (12). The wild-type enzyme ETT, mutant ETT-Ser^{25} and mutant ETT-Ser^{78}-Ser^{82} were differentially treated with DTT, HED, cystine, Cys-SO_3^-, and IAM as described in Experimental Procedures and analyzed by an isoelectric focusing gel, and the resulting pI values were examined (Fig. 27). Lanes 2 to 8 were the wild-type enzyme, ETT, treated with DTT, HED, cystine, Cys-SO_3^-, cystine/GSH, Cys-SO_3^-/GSH, and IAM, respectively; lanes 9 to 15 were mutant ETT-Ser^{25} incubated with DTT, HED, cystine, Cys-SO_3^-, Cys-SO_3^- /GSH, IAM, and IAA, respectively; and lanes 16 to 19 were loaded with cystine, Cys-SO_3^-, Cys-SO_3^-/GSH, and IAM treated mutant ETT-Ser^{78}-Ser^{82}, respectively. Usually, when treated with DTT or HED, the enzyme is in the reduced (thiol) or oxidized (intramolecular disulfide) form, respectively, and their pI values are widely different from each other (Fig. 27, lane 2 and 3, lane 9 and 10). However, when treated with cystine or Cys-SO_3^-, the pI values of ETT and ETT-Ser^{25} lay between those of their reduced and oxidized forms, i.e., the enzymes were neither in their thiol nor intramolecular disulfide forms, but rather, they may have been in partially mixed disulfide forms. In addition, more

Fig. 27 Isoelectric focusing analysis of the ES intermediates of thioltransferase. The wild-type enzyme, ETT, the mutants ETT-Ser^{25}, and ETT-Ser^{78}-Ser^{82} were separately treated with DTT, HED, cystine, Cys-SO_3^-, Cys-SO_3^-/GSH, cystine/GSH, and IAM as indicated with + (added) and - (not added). The pI value for each of the enzymes (either in the free state or in the modified state) was measured on a Servalyt Precoat isoelectric focusing gel, according to the manufacturer's instructions. The sample in each lane is indicated.


DTT
HED
Cystine
Cys-SO3
GSH
IAM
IAA
pI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
3.50
4.40
5.30
5.90
6.90
7.30
7.70
8.30
9.45
10.65
ETT
ETT-Ser25
ETT-Ser78Ser82

then one band existed in Cys-SO_3^- treated samples (lane 5 and lane 12), suggesting that both substrate components can form mixed disulfides with ETT and ETT-Ser^{25}. The mixed disulfide formed between ETT and GSH was also observed in the presence of disulfide substrates and GSH, and the pI was slightly different from that of the enzyme-half-disulfide substrate form. The pIs of IAM treated enzymes were quite similar to those of oxidized ones. This gave additional evidence that IAM reacted with Cys^{22} eliminating its negative charge. For mutant, ETT-Ser^{78}-Ser^{82}, the pI values were equal to that of the oxidized form when treated with cystine, Cys-SO_3^-, Cys-SO_3^-/GSH, and IAM. Thus, this mutant seems to favor the intramolecular disulfide form.

Radioactive Labeling Studies-- For further testing of the mixed disulfides between the enzyme and its substrates, two radioactive labeled substrates, [^{14}C]cystine and [^{3}H]GSH, were used to track the reaction progress. The details of the labeling experiments were described in Experimental Procedures and the results are listed in Table VII. As shown in Table VII, enzyme-substrate intermediates were detected, since radioactivity was measured in the collected protein fractions both before and after the concentration of the enzymes. The mutant ETT-Ser^{25} had the highest specific radioactivity (cpm/μg) both in [^{14}C]cystine labeled samples or in [^{3}H]GSH labeled samples. No counts were detected in mutant ETT-Ser^{22} due to the absence of the active site Cys^{22}. When labeled by [^{14}C]cystine, there were no counts detected in mutant ETT-Ser^{78}-Ser^{82}. This result agreed with that of the inhibition studies and suggested that an intramolecular disulfide was formed in this mutant. Pretreating ETT-Ser^{25} with IAM blocked [^{14}C]cystine labeling. This is additional evidence for the identification of Cys^{22} as

Table VII

Radioactive labeling of thioltransferases

The wild-type and mutant pig liver thioltransferases were incubated with [^{14}C-]cystine, [glycine-2-^{3}H]GSH, or Cys-SO_3^-/[^{3}H-]GSH in the presence of 100 mM sodium phosphate buffer, pH 7.5 for 20 min at room temperature, the excess radioactive labeled substrates were removed by Sephadex G-25, and the samples were concentrated under conditions described in the text. The radioactivity of each enzyme was counted by liquid scintillation spectrometry and the specific radioactivity was calculated.

Thioltransferases (0.3 mM)	Substrates			
	L-[^{14}C]cystine (0.6 mM)	[^{3}H]GSH (2.5 mM)	Cys-SO_3^- (12.5 mM)	+ [^{3}H]GSH (2.5 mM)
	cpm/μg	cpm/μg		cpm/μg
ETT	140	0		293
ETT-Ser^{22}	0	N.D.[a]		0
ETT-Ser^{25}	386	0		2020
ETT-Ser^{25} + IAM[b]	0	N.D.		N.D.
ETT-Val^{26}	74	N.D.		114
ETT-Gln^{27}	137	N.D.		242
ETT-Ser^{78}-Ser^{82}	0	N.D.		83

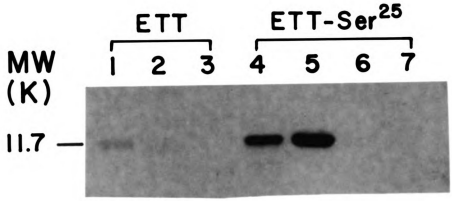
a N.D. Not Determined

b ETT-Ser^{25} was pretreated with IAM, then with L-[U-^{14}C]cysteine.

the active site, and alkylation at this position prevented labeling by either substrate. Except for the ETT-Ser^{22} mutant, all other tested enzymes were labeled by [^{3}H]GSH in the presence of Cys-SO_3^-. Direct incubation of ETT with [^{3}H]GSH, was unreactive, i.e., no isotope was incorporated into the enzyme. This result is also consistent with that of inhibition studies, i.e., GSH alone could not protect the enzyme against IAM inactivation. Thus, reduced enzyme must react with a disulfide substrate first, then with GSH to regenerate the dithiol or monothiol (ETT-Ser^{25}) form.

The wild-type enzyme, ETT, and the mutant ETT-Ser^{25} treated with the radioactive labeled substrates as described above were subjected to SDS-PAGE under nonreducing conditions and the dried gel was subjected to autoradiography (Fig. 28). Lanes 1 to 3 were ETT treated with [^{14}C]cystine, Cys-SO_3^-/[^{3}H]GSH, and [^{3}H]GSH, respectively, and lanes 4 to 7 were ETT-Ser^{25} treated with [^{14}C]cystine, Cys-SO_3^-/[^{3}H]GSH, and IAM followed by either [^{14}C]cystine, or [^{3}H]GSH, respectively. When treated with [^{14}C] cystine or Cys-SO_3^-/[^{3}H]GSH, strong labeling was found in the mutant ETT-Ser^{25} (Fig. 28, lane 4 and 5), indicating a covalent linkage between the enzyme and the substrate. With the same treatment, only faint signals were observed in ETT (Fig. 28, lanes 1 and 2) suggesting the formation of some intramolecular disulfide due to air oxidation during the electrophoresis process. No labeled bands could be seen when ETT-Ser^{25} was pretreated with IAM followed by [^{14}C]cystine, or when either enzyme was directly incubated with [^{3}H]GSH (Fig. 28, lanes 3, 6 and 7).

Fig. 28 Autoradiography of ETT and ETT-Ser^{25}. The wild-type (ETT) and mutant (ETT-Ser^{25}) thioltransferases were treated with radioactively labeled substrates, [^{14}C]cystine or [^{3}H]GSH, as described in Experimental Procedures. Lanes 1 to 3 were ETT treated with [^{14}C]Cystine, Cys-SO_3^-/[^{3}H]GSH, [^{3}H-]GSH, respectively, lanes 4 to 7 were ETT-Ser^{25} treated with [^{14}C]cystine, Cys-SO_3^-/[^{3}H]GSH, and IAM followed by [^{14}C]cystine, and [^{3}H]GSH, respectively.


ETT
ETT-Ser25
MW
(K)
1 2 3 4 5 6 7
11.7

DISCUSSION

Thiol-disulfide exchange reactions (Reaction 1) are actually nucleophilic ionic displacement reactions that take place either spontaneously or enzymatically, in vitro or in vivo (15,16). Askelöf, et al. first suggested the name, thioltransferase, for the enzyme that catalyzed these reactions (4 and 5), and was originally called a transhydrogenase. This model proposed that two GSH molecules consecutively replace the disulfide substrate (RSSR) to yield two reduced products (2 RSH) and one GSSG molecule. A RSSG intermediate was formed during the catalytic reaction process when R was an aryl moiety, but not when cystine was the disulfide substrate (7). How the enzyme was involved in this reaction was not considered in this model. According to one model, the formation of the mixed disulfide between the enzyme and GSH is unlikely since GSH always replaces the enzyme to form RSSG and leaves the enzyme in the reduced form.

$$E\text{-}S\text{-}S\text{-}R + GSH \rightleftharpoons R\text{-}S\text{-}S\text{-}G + E\text{-}S^- + H^+ \qquad (13)$$

However, this model can not fully explain the data presented in this paper. For example, the disulfide between enzyme and GSH was demonstrated, and after preincubation with Cys-SO_3^-/GSH, but not GSH, the enzyme was fully protected against IAM inhibition.

In contrast, our results do support other models (Reactions 6 to 9 and 10 to 12). As shown in the inhibition studies, IAM can inactivate the reduced enzyme by reaction with the ionized sulfhydryl group of Cys^{22}, but this inhibition can be variably prevented due to the formation of mixed- or intramolecular-disulfides after pretreating the enzymes with

disulfide substrates, but not the mutants without Cys^{25}. Preincubation of the enzymes with $Cys\text{-}SO_3^-$/GSH yielded much stronger protection. In addition, the enzymes could be labeled by [^{3}H-]GSH in the presence of $Cys\text{-}SO_3^-$ providing credence to Reactions 6-9 or 10-12. When treated with GSH or [^{3}H-]GSH alone, the enzymes could not be protected against IAM or be radioactively labeled. These data were consistent with the sequence of the reactions in the second model, i.e., disulfide substrate adds first to the reduced enzyme, then GSH.

The isoelectric focusing data are hard to explain according to Reaction 7. If an intramolecular disulfide is formed, the pI of the enzyme should be more alkaline and the enzyme would not be sensitive to inactivation by IAM. When treated with cystine, the wild-type enzyme (ETT) had a pI different from either the reduced form (treated with DTT) or oxidized form (treated with HED) and there are two such pI forms for the $Cys\text{-}SO_3^-$ treated enzyme (Fig. 27). One form, representing a mixed disulfide between enzyme Cys^{22} and cysteine, and the other may represent a S-thiol sulfate ester between Cys^{22} and $-SO_3^-$ derived from the substrate. ETT as well as ETT-Gln^{27} is still partially inhibited by IAM after the above treatments. In addition, exchange of Cys^{25} with Ser^{25} caused a 10% increase in thiol-disulfide exchange activity (11) and a 94% increase in DHA reductase activity (12). As an explanation, these results required an alternative mechanism from that shown by Reactions 7 and 8. For mutant, ETT-Ser^{78}-Ser^{82}, an intramolecular disulfide is formed, when treated with disulfide substrates. It has the same pI as that of the HED oxidized form and is not sensitive to IAM. Thus, we believe that, during the catalytic reactions, the formation of an intramolecular disulfide is an optional step dependent on the substrate

involved. In the absence of GSH, the enzyme catalyzes Reaction 6, and whether Reaction 7 proceeds or not depends on the strength of the S-nucleophilicity of the sulfhydryl group of Cys^{25} and the thiol of the first product, RSH (16). If the former is stronger, an intramolecular disulfide is formed (Reaction 7), whereas if the latter is stronger, the reaction will be equilibrated as in Reaction 6. In the presence of GSH, the reaction quickly proceeds to Reaction 8 directly from Reaction 6 or 7, then moves to Reaction 9 to complete a cycle. According to various results from this study and the considerations above, we suggested an alternative model (Reactions 10-12). This model represents a typical hexa-Uni Ping Pong mechanism, whereas the four step model (Reactions 6-9) is a Uni BiBiUni mechanism (17). The modified model may explain why the reaction catalyzed by the mutant ETT-Ser^{25} is more efficient than that catalyzed by the wild-type enzyme.

The mechanism of the DHA reductase activity of thioltransferase is not established. However, Cys^{22} is likely to be the active site for both intrinsic enzymatic activities, and the mechanism of DHA reductase activity is presumably similar to that of a thiol-disulfide exchange activity, i.e., a thio-hemiketal intermediate, instead of a mixed disulfide, followed by displacement with GSH (18).

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CONCLUSIONS

I started this dissertation by screening a pig liver cDNA library using polyclonal antibodies against pig liver thioltransferase (PLTT). A positive clone was sequenced and gave a deduced amino acid sequence identical to the directly determined one, except the N-terminus should be N-acetylalanine followed by a glutamine rather than the reverse as previously reported. The cDNA for PLTT was expressed at a high-level in E. coli JM105 in a soluble unfused active state. The establishment of the efficient PLTT expression system made it is possible to study the active site and catalytic mechanism of thioltransferase at a molecular level.

The replacements of selected amino acids at the active center of PLTT by site-directed mutagenesis techniques directly confirmed that Cys^{22} is the catalytic site for this enzyme. The mutation studies also revealed that the extremely low pK_a of the Cys^{22} sulfhydryl group (pK_a=3.8) is facilitated by Arg^{26}. The mutants Ser^{25} and Ala^{25} are totally different in enzyme activity, the former has 110% of the wild type enzyme activity, whereas the latter shows only 9% activity remained. This result established that an amino acid residue with a more hydrophilic side chain e.g., $-CH_2OH$ or $-CH_2-SH$, at position 25 is essential for optimal enzyme activity. The finding of the increased activity of the mutant Ser^{25} implied that the intramolecular disulfide bond between Cys^{22} and Cys^{25} is not necessary at least for this mutant enzyme catalytic mechanism, and led to the suggestion of an alternative three step catalytic pathway. This dissertation also directly demonstrated that the extra pair of cysteines, Cys^{78} and Cys^{82}, down-stream are not directly

involved in the enzyme catalytic action, but may have a structural function. The role of Lys^{27} was proposed to involve the stabilization the enzyme-substrate intermediate.

The success of cloning, sequencing and expression of PLTT opened a wider door for extending studies of its structure and function. Sufficient recombinant wild type enzyme has been made in mg quantities, and its crystallization and three-dimensional structure is under investigation in collaboration with Dr. Suresh K. Katti, Yale University. Similar studies using mutant enzymes will be carried out in the near future. Unquestionably, future results from high resolution X-ray analysis will be very helpful in answering remaining critical questions related to the structure and mechanism of thioltransferase. The mutant enzymes will also be used to survey the possible catalytic mechanism of dehydroascorbate reductase activity.

The cloned cDNA will be useful as follows: (1) as a probe to isolate the genomic DNA by screening a pig liver genomic DNA library, and sequencing the genomic DNA will reveal the splicing pattern of the thioltransferase gene; (2) to explore the thioltransferase distribution in tissues by Northern blot analysis; (3)in <u>in vivo</u> studies of the function of this enzyme by expressing it in transformed mammalian cell line and observing potential changes in transfected cells, such as in drug resistance; (4) as a probe to screen other mammalian cDNAs, e.g., human placenta and rat liver for sequence analysis and future transfection studies in tissue culture.