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William T. Wells
Major professor

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PURIFICATION, CHARACTERIZATION, PRIMARY STRUCTURE AND CATALYTIC MECHANISM STUDIES OF PIG LIVER THIOLTRANSFERASE

By

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ABSTRACT

PURIFICATION, CHARACTERIZATION, PRIMARY STRUCTURE AND CATALYTIC MECHANISM STUDIES OF PIG LIVER THIOLTRANSFERASE

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Thioltransferase functions as a GSH-disulfide oxidoreductase which can catalyze the reductive cleavage of a variety of disulfides, including protein disulfides. It has been suggested that thioltransferase could regulate various enzyme activities, in vivo, by modulating their thiol-disulfide status. The relationship between thioltransferase and a similar protein, glutaredoxin has been emphasized. The detailed studies of the structure and function of thioltransferase were undertaken.

By reversibly modifying the thiol-disulfide status of thioltransferase(T.Tase) and thereby causing a pI shift, a purification procedure for pig liver T.Tase was developed. The purified enzyme was demonstrated to be homogeneous by SDS PAGE, isoelectric focusing and HPLC. The protein has a molecular weight of 11,000, a native pI close to 6.4, and an amino acid composition similar to that of calf thymus glutaredoxin. The reaction catalyzed by the enzyme has an optimal pH of 9.0. The plots of T.Tase activity as a function of S-sulfocysteine, and GSH concentrations did not display Michaelis Menten kinetics. The complete amino acid

sequence of pig liver T.Tase was determined with either automated Edman degradation or Fast Atom Bombardment mass spectrometry. The enzyme is a single polypeptide with 105 amino acid residues and an acetylated glutamine N-terminus. The protein has two cysteine pairs with sequences of -Cys-Pro-Phe-Cys- and -Cys-Ile-Gly-Gly-Cys-, respectively. The sequence of the pig liver enzyme showed 82% homology with calf thymus glutaredoxin, suggesting they are the same enzyme from two different species. The active site cysteine of the enzyme was identified as Cys 22. The kinetics of the reaction between Cys 22 of the reduced enzyme and iodoacetamide as a function of pH revealed that the active site sulfhydryl group had a pKa of about 3.8. A catalytic mechanism for thioltransferase was suggested by studies of isotopic labeling and by the kinetics of the reactions between active site cysteine and the substrates.

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ABBREVIATION	
CAD	collisional activation dissociation
CNBr	cyanogen bromide
EDTA	ethylenediaminetetraacetic acid
FAB	fast atom bombardment
FPLC	fast protein liquid chromatography
GSH	reduced glutathione
GSSG	oxidized glutathione
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
M_r	relative molecular mass
PAGE	polyacrylamide gel electophoresis
PAS	periodic acid-Schiff
pI	isoelectric point
PTH	phenylthiohydantoin
SDS	sodium dodecylsulfate
Tris	tris(hydroxymethyl)aminomethane
T.Tase	thioltransferase

INTRODUCTION

The initial goal of this Ph.D. program was to explore the physiological role of thioltransferase. When I started my research in the spring of 1984, Drs. William W. Wells and Jeffrey Nickerson found that rat liver thioltransferase could apparently stimulate NDP kinase activity. To continue this work, I undertook the activation mechanism studies. Since the thioltransferase used at that time was an impure preparation, I had to develop a purification procedure for the homogeneous enzyme. Rat liver thioltransferase was purified to homogeneity and partially characterized. The purification procedure was described in Journal of Biological Chemistry, 261, 996-1001(1986). With the homogenous preparation, rat liver thioltransferase prevented inactivation of NDP kinase. However, when the thioltransferase was inactivated by iodoacetamide, it still could prevented NDP kinase inactivation. It turned out that other proteins, such as hexokinase and bovine serum albumin, could also prevent NDP kinase inactivation. Thus thioltransferase played only a general protective role, not one of specific activation.

During the course of purification and characterization of rat liver thioltransferase, several interesting results emerged. The enzyme activity was very sensitive to

iodoacetamide, suggesting that the active site of the enzyme contained cysteine; When the purified rat liver thioltransferase was run on an isoelectric focusing gel, doublet bands were detected, but the higher pI band disappeared upon adding dithiothreitol; The amino acid composition of rat liver thioltransferase was similar to that of calf thymus glutaredoxin. Further exploration of these initial observations became the basis of my Ph.D thesis project. Since only limited numbers of rat livers were available, pig liver thioltransferase was used in the structural and catalytic mechanism studies. A new purification procedure for pig liver thioltransferase which took advantage of the marked pI shift of the enzyme, treated with dithiothreitol and hydroxyethyl disulfide, permitted the preparation of relatively large quantities of homogeneous enzyme. This work was published in Analytical Biochemistry 162, 265-273 (1987), and the manuscript is reproduced in Chapter III. The complete amino acid sequence of pig liver thioltransferase is described in Chapter IV, which was published in the Journal of Biological Chemistry 262, 6699-6703 (1987). The primary structure of pig liver thioltransferae allowed me to identify and characterize its active site. These results were published in the Journal of Biological Chemistry 262, 6704-6707 (1987) and this manuscript constitutes part of Chapter V.

CHAPTER I

LITERATURE REVIEW

The effect of thiol-disulfide interchange on enzyme activity is an accepted part of the biological repertoire. The reversible covalent modification of the sulfhydryl groups of an enzyme can result from either the formation of a protein mixed disulfide (equation 1);

$$EnzSH + RSSR \rightleftharpoons EnzSSR + RSH \quad (1)$$

or through an intramolecular disulfide (equation 2).

$$Enz(SH)_2 + RSSR \rightleftharpoons EnzS_2 + 2RSH \quad (2)$$

The properties of several enzymes have been shown to be affected by thiol-disulfide exchange (1). For example, rabbit liver fructose 1,6-bisphosphatase (2), glucose 6-phosphatase (3), δ-aminolevulinate synthetase (4) and spinach acid phosphatase (5) are stimulated directly or indirectly by reaction with disulfides. In contrast, phosphofructokinase (6,7), pyruvate kinase (8), hexokinase (9,10), glycogen synthase I and D (11,12), adenylate cyclase (13,14), 3-hydroxy-3-methylglutaryl coenzyme A reductase (15,16), and phosphorylase phosphatase (17) are inactivated upon treatment with GSSG or other biological disulfides. Although the above correlations between thiol:disulfide ratios and enzyme activities support the hypothesis that this form of protein modification may take part in cellular enzyme regulation, direct experimental support for the coupling of the activity to the thiol:disulfide ratio, <u>in vivo</u>, is generally lacking. On the other hand, the reaction rate of a thiol:disulfide

interchange is markly affected by the cellular redox state and is usually quite slow at physiological pH (1). The low reaction rates and lack of specificity of modification of protein thiol:disulfide status by low molecular thiols and disulfides led people to search for enzymes which can catalyze the reaction of protein-thiol:disulfide interchange. The most likely candidates for such systems are membrane-bound thiol:protein-disulfide oxidoreductase (EC 5.3.4.1) and protein:disulfide isomerase (EC 1.8.4.2), and cytosolic thioredoxin, glutaredoxin, and thioltransferase.

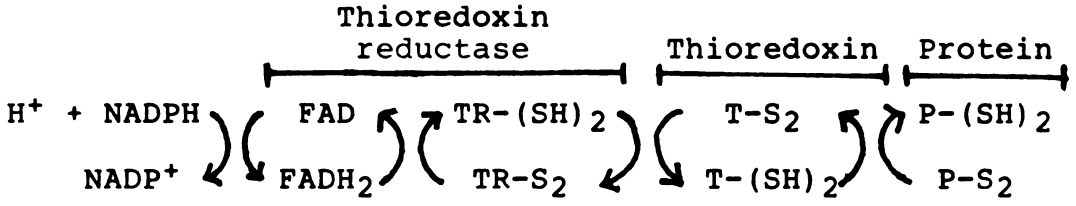
Thiol:protein-disulfide oxidoreductase, also known as glutathione-insulin transhydrogenase, and protein:disulfide isomerase activities were discovered more than two decades ago (18-21). They have been identified in all animal tissues studied and in plant material (22). Because of the coexistence of thiol:protein-disulfide oxidoreductase and protein:disulfide isomerase activities in various reported preparations, there has been considerable controversy as to whether thiol:protein-disulfide oxidoreductase and protein:disulfide isomerase represent one, two, or possibly several different proteins (22). Recently, immunological properties of these enzymes from four different laboratories were compared (23). The immunological identity in double immunodiffusion and rocket-line immunoelectrophoresis suggested that the two enzymes classified before represent the same enzyme with alternative activities. Earlier reports

of differences in physicochemical properties between different preparations of the enzyme may be due to the differences in the extent of modification and/or degradation during different purification procedures. The two enzymes are located in the endoplasmic reticulum, and they appear to be associated with the luminal face of the membrane (24-26). It turns out that the precise activities of the enzymes depend on the protein substrate and the conditions (27). With a reduced protein substrate in the presence of dissolved oxygen, a GSSG/GSH mixture, etc., the enzyme catalyzes the formation of native protein disulfide. With an oxidized protein substrate containing non-native disulfide bonds, the enzyme catalyzes the rearrangement of the "scrambled" protein. However, with an oxidized protein substrate under strong reducing condition (10 mM GSH), the overall reaction will be a net reduction of protein disulfide. It is believed that thiol:protein-disulfide oxidoreductase or protein disulfide isomerase play important roles in the degradation of disulfide-containing peptide hormones (28,29) and native disulfide formation in protein synthesis (27). However, no evidence has shown that they can regulate the activities of other enzymes by catalyzing thiol-disulfide interchange.

Three cytosolic proteins, thioredoxin, glutaredoxin, and thioltransferase, have received increasing attention as a regulatory protein by changing thiol-disulfide status of metabolic enzymes.

Thioredoxin is the most intensively studied protein among these three proteins. It was first identified as the hydrogen donor for the enzymatic synthesis of deoxyribonucleotide by ribonucleotide reductase in E. coli (30). Research on the structure and function of thioredoxin has since then turned out to be fruitful. Structure studies revealed that thioredoxins from bacteria, yeast, plant and animal cells have a molecular weight around 12,000 kDa and have evolved from a common ancestor (31). The active sites of thioredoxin from E. coli and yeast are dithiols with a common sequence of -Cys-Gly-Pro-Cys- (32,33) Thioredoxin from E. coli was crystallized in 1970 (34). The three dimensional structure solved by X-ray crystallographic methods to 2.8 angstrom resolution showed that the active site disulfide bridge was located in a protruding part of the three dimensional structure (35). It has been found that the thioredoxin systems (thioredoxin and NADPH-thioredoxin reductase) is involved in a wide variety of biochemical processes. It functions as a hydrogen donor in several enzyme systems, such as ribonucleotide reductase, and methionine sulfoxide reductase (36,37). It is well known that plant thioredoxin is involved in electron transfer in photosynthetic cells (38). Thioredoxin is also essential for viral DNA polymerase and the assembly of single strand DNA phage f1 (39,40). More interestingly, it has been shown that many enzyme activities are regulated by thioredoxin. In

plants, reduced thioredoxin activated certain chloroplast enzymes, such as fructose 1,6-bisphosphatase, NADP-malate dehydrogenase, and phosphoribulokinase (41,42). In mammalian cells, regulation of the activity of glucocorticoid receptor and of initiation of protein synthesis in reticulocytes by the thioredoxin system have been demonstrated (43,44). Thioredoxin is also a general protein disulfide reductase. The electron transfer pathway proposed by Holmgren is as follows (31):



The reduction mechanism of protein disulfide by thioredoxin involves the reversible transfer of electrons from NADPH to protein disulfide via redox reactive dithiols located at the active sites of thioredoxin reductase and thioredoxin (31). The ability of thioredoxin to change protein thiol-disulfide status implied that thioredoxin may play a role in the regulation of enzyme activity by thiol-disulfide control.

Analysis of an E. coli mutant lacking thioredoxin but with fully active NADPH-dependent deoxyribonucleotide synthesis led to the discovery of a new hydrogen transport system consisting of NADPH, GSH, GSSG reductase, and a heatstable protein called "glutaredoxin" (45). Later on, the first evidence for a mammalian glutaredoxin was obtained in

calf thymus (46). Glutaredoxin also has GSH-disulfide oxidoreductase, or transhydrogenase activity in a coupled system with NADPH and glutathione reductase. Small disulfides, such as hydroxyethyl disulfide, cystine, and S-sulfocysteine, are about equally good as substrates for both E. coli and calf thymus glutaredoxin while insulin is not a good substrate (47). Although glutaredoxin has only a 10 year history, it is chemically well characterized. Glutaredoxin from E. coli contains 85 amino acid residues with an active center sequence of -Cys-Pro-Tyr-Cys- located between positions 11 and 14 (48). The gene for E. coli glutaredoxin has recently been cloned and overproduced (49). The calf thymus glutaredoxin has 101 amino acid residues. The protein contains two cysteine pairs, -Cys-Pro-Tyr-Cys- and Cys-Ile-Gly-Gly-Cys-. The first cysteine pair was proposed to be the active site of the protein (50).

The relative contribution of thioredoxin and glutaredoxin to ribonucleotide reduction in normal cells is unknown. The apparent concentration of thioredoxin in wild type E. coli is about 100 fold higher than that of glutaredoxin (51). However, when these two proteins were coupled to the ribonucleotide reductase system, the molecular activity of glutaredoxin is about 10 fold higher than that of thioredoxin (51). The normal growth of cells lacking detectable thioredoxin shows that the glutaredoxin pathway is sufficient to support ribonucleotide reductase activity.

While those working on thioredoxin and glutaredoxin were intensively studying their structures and function in the ribonucleotide reductase system, another group of investigators put their emphasis on the possible regulatory role of thioltransferase in cellular metabolism. Compared with thioredoxin and glutaredoxin, thioltransferase has a long history. More than 30 years ago, thioltransferase was first identified in beef liver by Racker (52). In the course of the studies on the reduction of oxidized glutathione by beef liver glutathione reductase, it was found that a number of other disulfides, including homocystine, were reduced in the presence of crude liver extracts. However, the reaction rate of homocystine reduction proceeded quite slowly as compared with glutathione reduction. Analysis of the homocystine reduction system revealed a new enzyme which catalyzed the hydrogen transfer from GSH to a disulfide compound (52). The enzyme was given the name, glutathione-homocystine transhydrogenase. This enzyme activity has subsequently been found in bovine kidney (53), yeast (54), and rat liver (55). All the different preparations of the enzyme catalyze the reduction of low molecular weight disulfides by GSH coupled with NADPH and glutathione reductase:

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

$$GSSG + NADPH + H+ \xrightleftharpoons{\text{GSSG Reductase}} 2GSH + NADP+ \quad (2)$$

In 1970, Askelöf et al. found that the essence of the reaction catalyzed by the so called "GSH-disulfide transhydrogenase" was the transfer of a thiol group in a two step reaction:

$$RSSR + GSH \rightleftharpoons RSSG + RSH \quad (1)$$

$$RSSG + GSH \rightleftharpoons GSSG + RSH \quad (2)$$

GSH is regenerated by glutathione reductase system. They suggested a name of thioltransferase (56). Since then, the name of thioltransferase has been accepted by most laboratories. Thioltransferase from rat liver and bovine liver has been purified to apparent homogeneity and characterized to some extent (57,58). The enzyme has a molecular weight of approximately 11,000 kDa. A variety of disulfides, including protein disulfides, can be used as a substrate of the enzyme. The enzyme does not act as a protein:disulfide isomerase, as measured by reactivation of "scrambled" ribonuclease and Kunitz soybean trypsin inhibitor (58). Thioltransferase was found in various rat and bovine tissues (58,59). Primary results of subcellular distribution of thioltransferase showed that the cytosolic fraction contained the major enzyme activity (60). The enzyme activity was also detected in the lysosome rich fraction but no detectable activity was found in the microsome and mitochondria fractions (60). The physiological role proposed

for thioltransferase has been based on its ability to reduce protein disulfides in the presence of GSH. By treating cytosolic proteins with ^{35}S glutathione disulfide to form mixed disulfide of protein and glutathione, Mannervik and Axelsson were able to demonstrate that rat liver thioltransferase was essential in protein mixed disulfide reduction (61,62). The spontaneous reduction of protein mixed disulfide by GSH was negligible in comparison with the enzymatic reaction. GSH is the principal low molecular weight thiol in biological systems and it has been thought that GSH may function as a reductant of protein disulfide (63). However, the results above do not support this possibility because of the negligible spontaneous reaction rate. Using GSH as a substrate, thioltransferase may function as a general protein disulfide reductase to keep various cellular proteins in the reduced state. The activity of oxidized inactive papain could be restored to 85% of the original activity by adding thioltransferase (64). Pyruvate kinase from liver is inactivated by thiol oxidation processes (8). Reduced glutathione protects the enzyme against such inactivation, but even concentration as high as 10 mM do not afford full protection. However, the thioltransferase system including GSH, NADPH, and glutathione reductase, gave full protection of pyruvate kinase against oxidative inactivation (65).

More recently, a cytosolic protein, soluble protein

factor (SPF), was found to promote the GSH-dependent iodothyronine 5' deiodinase activity in rat kidney microsome fractions (66,67). According to the purification procedure, molecular properties, and the ability to function as a GSH-disulfide oxidoreductase, SPF and thioltransferase may be identical proteins (66).

Despite much progress in defining the enzymes involved in thiol-disulfide interchange of proteins, many important questions remain. For example, we need to learn the relative contribution of each enzyme to modulate protein thiol-disulfide status. It seems that the two membrane-bound enzyme activities, thiol:protein disulfide oxidoreductase and protein:disulfide isomerase, are responsible for some protein degradation and native disulfide formation in protein synthesis while the three cytosolic proteins, thioredoxin, glutaredoxin and thioltransferase, are involved in reducing equivalent transfer and thiol-disulfide interchange of biological molecules. Nevertheless, the relation among these three cytosolic proteins is not clear. Glutaredoxin appears to be different from thioredoxin since glutaredoxin can not be coupled to the NADPH-thioredoxin reductase system to catalyze ribonucleotide reduction and thioredoxin can not take part in the GSH-dependent ribonucleotide reduction (68). On the other hand, the abilities of the thioredoxin and thioltransferase systems to reduce low molecular disulfides and protein disulfides are different (69). Low molecular

weight disulfides are preferentially reduced by the thioltransferase system, while the relative important of each system in the reduction of protein disulfides depends upon the nature of the substrate. Whether glutaredoxin and thioltransferase represents the same enzyme or not needs further study. Both proteins catalyze the reduction of low molecular weight disulfides, but the similarity and difference of their structure and ability to be coupled to ribonucleotide reduction system remain unknown. If thioltransferase can donate hydrogen to the ribonucleotide reductase system, this will contribute significantly to clarification of the relationship between glutaredoxin and thioltransferase. Since amino acid sequences of thioredoxin and glutaredoxin have been previously determined, primary structure studies of thioltransferase will definitely help elucidate the identity and function of these proteins. Although thioltransferase was found more than 30 years, the chemical characterization of this enzyme was hampered by lengthy, low-yielding purification procedure (57,58). Developing a rapid and high-yielding purification procedure for thioltransferase was essential for further structural studies.

The catalytic mechanism of thiol-disulfide exchanging enzymes is a challenging field. Carmichael et al. have demonstrated that thiol:protein-disulfide oxidoreductase is reactive with iodoacetamide only after initial exposure to

its sulfhydryl substrate, GSH (70). Once the enzyme was transferred to a reactive state by thiols, thiol:protein-disulfide oxidoreductase will readily react with iodoacetamide, resulting in complete loss of catalytic activity. By reversible conversion of the redox state of the enzyme by GSH and GSSG, they were able to show that alkylation of the two cysteines of the enzyme led to inactivation of the enzyme. However, the location of these two cysteines was not determined, nor was it established whether one or two of these cysteines participate in the catalytic event. The amino acid sequence of protein:disulfide isomerase derived from the cDNA sequence showed two distinct regions homologous with E. coli thioredoxin (71). Each of these regions contains a sequence, -Trp-Cys-Gly-His-Cys-Lys-, which is similar to the active site sequence of E. coli thioredoxin. Whether or not these sequences represent the active site of protein disulfide isomerase needs to be confirmed. If thiol:protein-disulfide oxidoreductase and protein isomerase are the same enzyme, the two reactive cysteines Carmichael identified could be located on one or two of these sequences.

Great progress in elucidation of the catalytic mechanism of E. coli thioredoxin has been made by the Holmgren group (32,33). The active center residues of E. coli thioredoxin, Cys-32 and Cys-35, are joined by a disulfide bridge in the oxidized form (31). Reduction of this bridge will cause a

localized conformational change (72,73). The modification of cysteine residues in E. coli thioredoxin(SH_2), by alkylation with [^{14}C]iodoacetic acid, led to labelling of only Cys-32 below pH 8 (74). The reaction is strongly pH dependent. The pKa values of Cys-32 and Cys-35 are 6.7 and 9, respectively. A mechanism of action for protein disulfide reduction by thioredoxin was proposed (74), though not yet experimentally proven. According to the mechanism, Cys-32 initiates a nucleophilic attack on a protein disulfide substrate, resulting in a mixed disulfide intermediate between the protein and thioredoxin. The next step is a rapid intramolecular rearrangement initiated by deprotonation of Cys-35 followed by a nucleophilic attack of this thiolate on the mixed disulfide generating oxidized thioredoxin and substrate dithiol.

Compared with thioredoxin, little is known regarding the catalytic mechanism of thioltransferase and glutaredoxin. Since GSH is a major intracellular thiol and these two proteins interact with GSH to reduce a variety of physiological disulfides, the catalytic studies of these two proteins could facilitate a clearer understanding of the role of reversible enzyme thiol-disulfide formation in metabolism.

There is no doubt that changes of thiol-disulfide status of enzymes will often affect enzyme activity, and that biological thiols and disulfides can change this status. However, general lack of specificity required for a

biological control mechanism is the major problem when considering low molecular weight thiol and disulfide as effectors. It has been well established that the regulation of numerous enzymes by phosphorylation and dephosphorylation proceeds by way of the differential activities of protein kinases and phosphatase (75). Is there a similar system, *in vivo*, that can reversibly generate the thiol-disulfide interchange of certain enzymes, and thereby regulate their activities? Although many reports have suggested that thioredoxin and thioltransferase may play such a role, direct evidence for this process is difficult to obtain. It will be useful to further explore the potential interaction of these soluble proteins with other enzymes of metabolic interest.

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CHAPTER II

PURIFICATION AND CHARACTERIZATION OF PIG LIVER THIOLTRANSFERASE

ABSTRACT

An enzyme catalyzing thiol-disulfide exchange, thioltransferase, has been purified to homogeneity from pig liver. By taking advantage of the relatively large pI shift of the enzyme between its reduced and disulfide forms, the purification procedure, which included a heat step, ammonium sulfate precipitation, Sephadex G-75 and G-50 gel chromatography, and two CM-Sepharose chromatography separations, resulted in homogeneous protein with a 32% overall yield. Homogeneity was demonstrated by SDS polyacrylamide gel electrophoresis, isoelectric focusing, and high performance liquid chromatography. The protein has a Mr of approximately 11,000 and a pI for the reduced enzyme close to 6.4. The amino acid composition of the enzyme is similar to that of rat liver thioltransferase and calf thymus glutaredoxin, and the N-terminus of the protein was blocked. The optimal pH for the enzyme activity was 9.0. The plots of thioltransferase activity as a function of S-sulfocysteine, 2-hydroxyethyl disulfide, and reduced glutathione concentrations did not display Michaelis-Menten kinetics. The enzyme was very sensitive to sulfhydryl alkylating reagent. Preincubation of the enzyme with its disulfide substrates prevented the enzyme from inactivation by iodoacetic acid while the other substrate, GSH, did not

provide such protection. The results suggest that the active center of thioltransferase is cysteine dependent.

INTRODUCTION

Thioltransferase was first identified in rat liver by Racker who named the enzyme, glutathione-homocystine transhydrogenase (1). Since the enzyme can catalyze the reductive cleavage of a variety of disulfides including protein disulfides (2-4), it has been suggested that thioltransferase could regulate various enzyme activities, *in vivo*, by modulating their thiol-disulfide status (2,5). As a example, *in vitro*, the rate of reactivation by reduced glutathione of pyruvate kinase that had previously been inactivated nonenzymatically by glutathione disulfide was significantly increased by thioltransferase (6,7). Recently, a purified protein from rat liver cytoplasm has been shown to promote GSH-dependent iodothyronine 5'- deiodinase activity (8). The protein also exhibited both thioltransferase activity and glutaredoxin activity as assayed by activation of ribonucleotide reductase in the presence of GSH. The similarities between thioltransferase and glutaredoxin have been recently discussed (8,9).

Rat liver and bovine liver thioltransferase have been purified to apparent homogeneity (3,10), but the procedures were lengthy and had low yields(3-5%). Because of my interest in the structure and catalytic mechanism of thioltransferase, I have undertaken the development of a new

scheme that allows the rapid purification of relatively large quantities of the enzyme. A simple procedure with high yield was recently developed for rat liver thioltransferase which has a pI of 8.8 (9). Since only a limited amount of rat liver was available, I applied the same procedure to calf and pig liver thioltransferase without initial success because they have a more neutral pI.

In this article, I describe a new purification procedure for pig liver thioltransferase based on reversible modification of the protein by dithiothreitol and 2-hydroxyethyl disulfide. This modification caused a marked shift in its pI from 6.4 to approximately 8.0 and resulted in the subsequent purification of thioltransferase from pig liver to homogeneity in high yield (32%). Accordingly, I report the characterization of pig liver thioltransferase.

EXPERIMENTAL PROCEDURES

Materials

Sephadex G-25, G-50, and G-75 were purchased from Pharmacia Fine Chemicals. Acrylamide, N, N-methylenebisacrylamide, ammonium persulfate, protein molecular weight markers, and SDS were from Bio-Rad Laboratories. CM-Sepharose, glutathione reductase, NADPH, 2-hydroxyethyl disulfide, reduced glutathione, dithiothreitol, and iodoacetic acid were purchased from Sigma. S-sulfocysteine was prepared as described previously (11). Serva Blue W and Servalyt Precote isoelectric focusing gels were purchased from Serva Fine Biochemicals, Inc. . Isoelectric focusing marker proteins were purchased from BDH Chemicals, Ltd., Poole England. A C18 reversed phase HPLC column was from Varian. Acetonitrile and trifluoroacetic acid were HPLC grade and from J. T. Baker Chemical Co. and Pierce. Pig livers(4-6 months old) were obtained from the Michigan State University Meat Laboratory. All other chemicals and reagents were analytical grade.

Methods

Thioltransferase Assay-The enzyme was assayed as

described previously (9). Briefly, the assay mixture consisted of 0.5 mM GSH, 1.2 U of glutathione reductase, 2.5 mM S-sulfocysteine, 0.35 mM NADPH, 0.137 M sodium phosphate buffer, pH 7.5, and enzyme to be assayed in a volume of 0.5 ml. The reaction proceeded at 30° C and thioltransferase activity was measured spectrophotometrically at 340 nm. One unit of thioltransferase was defined as that amount of enzyme which catalyzes the formation of a umole of GSSG per min under standard conditions.

Amino Acid Composition Analysis-The homogeneous pig liver thioltransferase was carboxymethylated as described by Gracy(12). The amino acid hydrolysis of carboxymethylated pig liver thioltransferase was carried out with constant boiling HCl in sealed, evacuated reaction vial at 110° C for 24 hours. Amino acids were determined as phenylisothiocyanate (PITC) derivatives as described (13).

N-terminus Determination-Carboxymethylated pig liver thioltransferase (3-5 nmol) was used to determine the N-terminus by automated Edman degradation on a Beckman Model 890M sequencer. Degradation was conducted for up to 5 cycles.

Protein Determination-Protein was determined by the method of Lowry et al.(14) with bovine serum albumin as the standard.

SDS Polyacrylamide Gel Electrophoresis-For routine SDS polyacrylamide gel electrophoresis, the Laemmli system (15)

was employed. The stacking and separating gels (0.75 mm) had polyacrylamide concentrations of 6% and 15% respectively. The gels were run at a constant current of 15 mA for stacking gel and 20 mA for separating gel in a Hoefer SE 600 electrophoresis apparatus. The gels were stained with Serva Blue W.

Isoelectric Focusing-A Servalyt Precote isoelectric focusing gel, pH 3-10, was used. Samples were run at constant power of 1 W with an LKB Ultraphore isoelectric focusing apparatus. The gels were stained by Serva Blue W.

Purification of Pig Liver Thioltransferase-Livers (2 Kg) from 4-6 month old pigs were used as starting material for the purification. The purification procedures included preparation of a high speed supernatant, a heat step, ammonium sulfate fractionation, Sephadex G-75 gel filtration, Sephadex G-50 gel filtration, the first CM-Sepharose chromatography, and the second CM-Sepharose chromatography. The first four steps from the high speed supernatant preparation through Sephadex G-75 gel filtration were the same as those employed in the purification of rat liver thioltransferase (9). The remaining steps are described as follows.

Sephadex G-50 Gel Filtration-The thioltransferase fractions from the Sephadex G-75 (Fig 1) were pooled (about 450 ml). The soluble proteins were concentrated by precipitation at 70% saturated ammonium sulfate. The

concentrated proteins dissolved in water (about 40 ml) were incubated with 5 mM dithiothreitol for 15 min at room temperature, and then loaded on a Sephadex G-50 column (3 x 110 cm) equilibrated and eluted with 10 mM sodium phosphate buffer, pH 6.1 (Fig 2).

The First CM-Sepharose Chromatography-Thioltransferase fractions (about 65 ml in 10 mM sodium phosphate buffer, pH 6.1) from the Sephadex G-50 gel filtration (Fig. 2) were incubated with 5 mM dithiothreitol for 15 min at room temperature, and then loaded on a CM-Sepharose column (2.5 x 25 cm) equilibrated with 10 mM phosphate buffer, pH 6.1. The column was first washed with 50 to 100 ml of 10 mM sodium phosphate buffer, pH 6.1, and the enzyme was eluted with 10 mM sodium phosphate buffer, pH 7.0. The thioltransferase fractions(about 60 ml) were pooled and lyophilized.

The Second CM-Sepharose Chromatography-The lyophilized enzyme from the first CM-Sepharose column was incubated with 10 mM dithiothreitol for 15 min at 30° C. The incubation mixture was loaded onto a Sephadex G-25 column equilibrated and eluted with 10 mM sodium phosphate buffer, pH 6.4. The thioltransferase fractions (20 drops per tube) were directly collected into test tubes which contained 0.1 ml of 20 mM 2-hydroxyethyl disulfide. Thioltransferase fractions were pooled and loaded onto a CM-Sepharose column (1.5 x 28 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.4. The column was first washed with 50 to 100 ml of 10 mM phosphate,

pH 6.4, and then eluted by a linear gradient formed by mixing 80 ml of 10 mM sodium phosphate buffer, pH 7.0, with 80 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The peak of thioltransferase activity was eluted at a concentration of 45 mM NaCl (Fig. 3). The thioltransferase fractions of the CM-Sepharose chromatography were lyophilized and reduced by 10 mM dithiothreitol before desalting on a Sephadex G-25 column.

RESULTS

Purification of the Enzyme-Thioltransferase from 2 Kg pig liver was purified 1490 fold with a yield of 32% (Table 1). In the early steps from acid precipitation to ammonium sulfate precipitation, I was able to remove much contaminating protein and to provide a relatively small loading volume for the subsequent gel filtration. After the two sizing columns, Sephadex G-75 and G-50 chromatography (Figs 1 and 2), almost all proteins above 20,000 kDa were separated from thioltransferase as detected by SDS polyacrylamide gel electrophoresis (data not shown). When I purified rat liver thioltransferase (9), we found that the enzyme has two pI forms differing by 0.5 pH units. The higher pI form was apparently converted into the lower pI form when the enzyme was incubated with dithiothreitol. The same property was found for pig liver thioltransferase during the purification. I was able to convert the enzyme to a lower pI form by treatment with dithiothreitol and to a higher pI form by treating the reduced enzyme with 2-hydroxyethyl disulfide. The difference between the two pIs was more than one pH unit. Thus, by rechromatography of the two different pI forms of the enzyme on the same CM-Sepharose column at different pHs, the enzyme was purified to homogeneity (Fig 3).

TABLE 1

Purification of pig liver thioltransferase[1]

Purification step	Protein (mg)	T.Tase[2] activity (units)	Specific activity (units/mg)	yield (%)	Purification (fold)
High speed supernatant	111,750	10,060	0.09	100	1
Heat and ammonium Sulfate precipitation	35,280	9,090	0.26	90	3
Sephadex G-75	4,000	7,450	1.9	74	21
Sephadex G-50	461	5,650	12	56	133
First CM-Sepharose	81	3,950	49	39	544
Second CM-Sepharose	24	3,220	134	32	1490

[1] 2 kilograms of liver from 4 to 6 month old pig.
[2] Thioltransferase.

Fig 1. Sephadex G-75 gel filtration of pig liver thioltransferase. ▲-----▲: Absorbance at 280 nm. ●-----●: Thioltransferase activity.

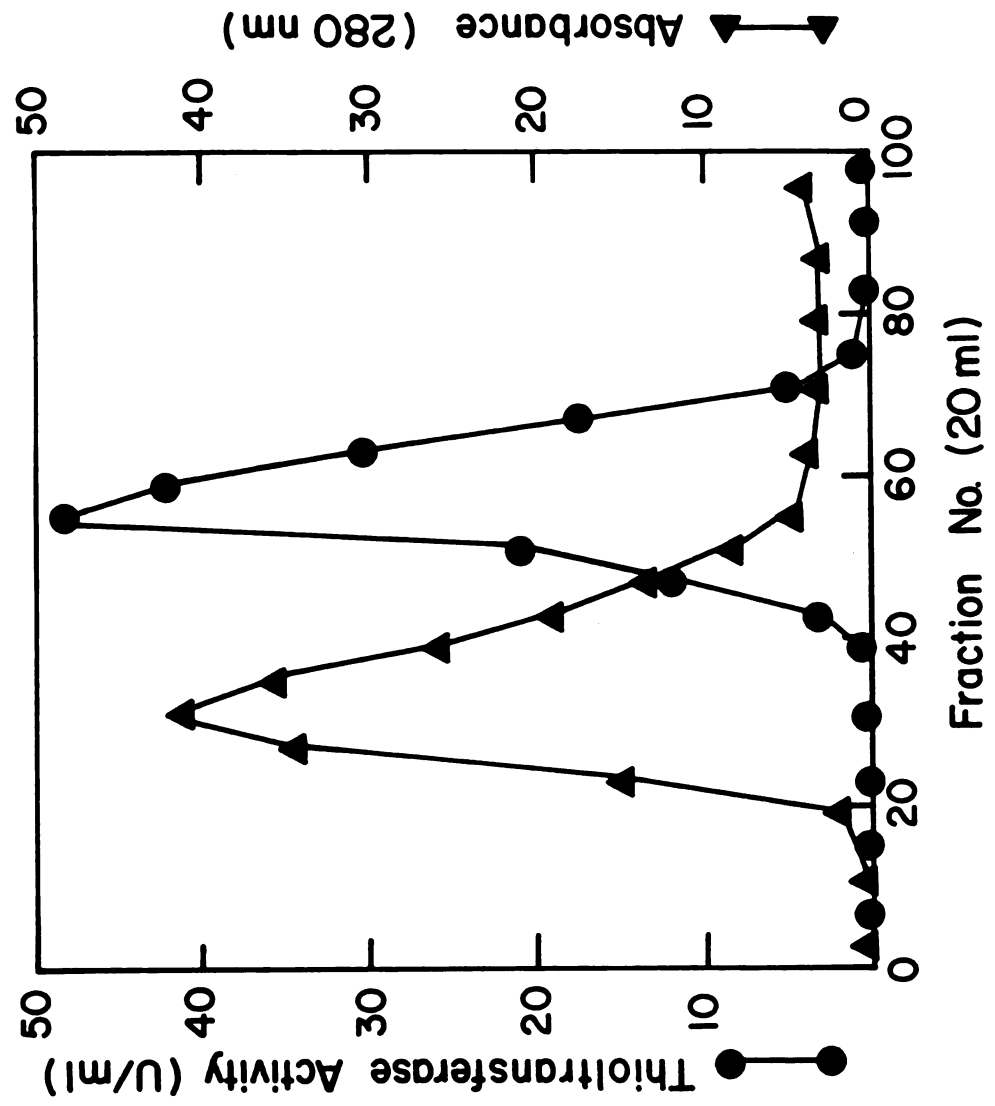

Thioltransferase Activity (U/ml)
Absorbance (280 nm)
Fraction No. (20 ml)
0
10
20
30
40
50
0
20
40
60
80
100

Fig 2. Sephadex G-50 gel filtration of pig liver thioltransferase. ▲-----▲ : Absorbance at 280 nm. ●-----● : Thioltransferase activity.

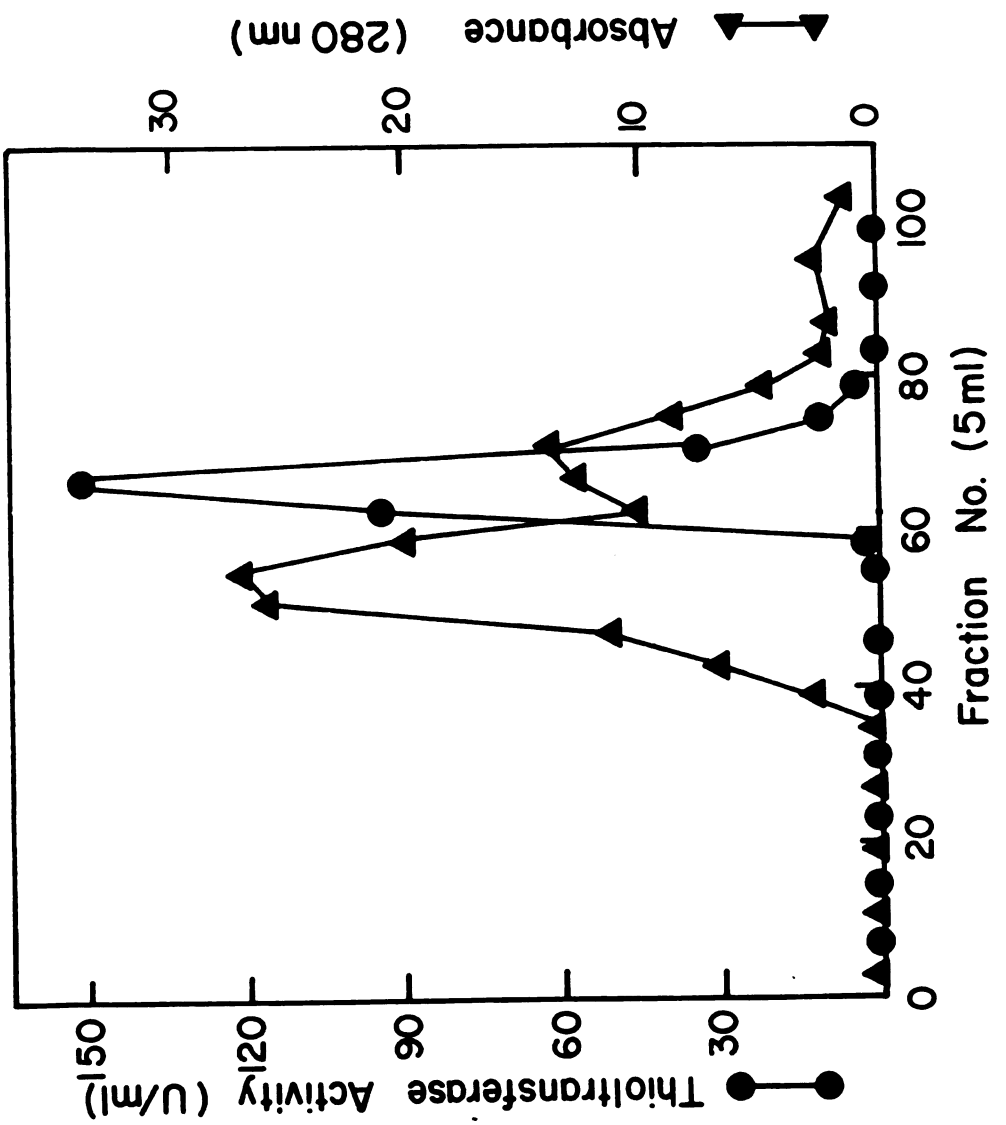
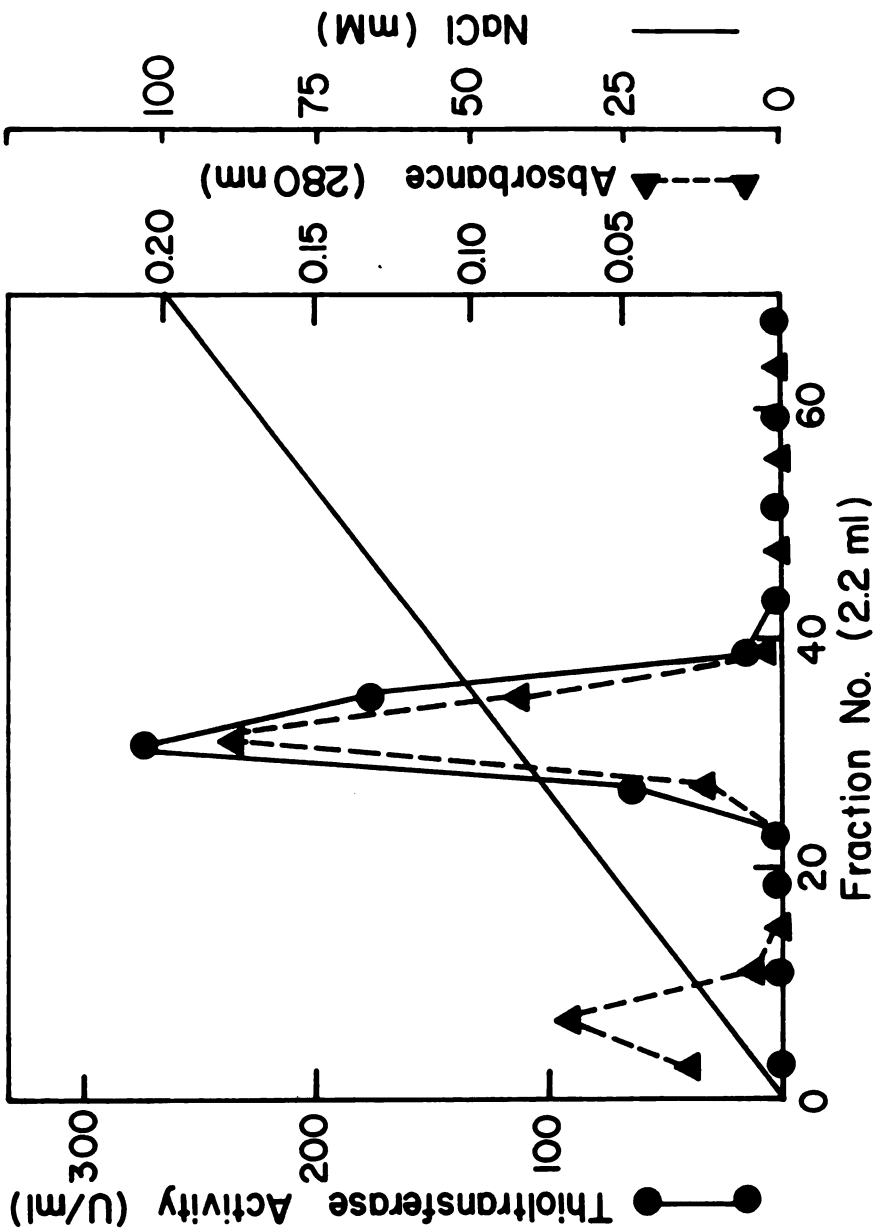

Thioltransferase Activity (U/ml)
150
120
90
60
30
0
20
40
60
80
100
Fraction No. (5ml)
30
20
10
0
Absorbance (280 nm)

Fig 3. Second CM-Sepharose chromatography of pig liver thioltransferase. ▲-----▲ : Absorbance at 280 nm. ●-----● : Thioltransferase activity.


Thiolltransferase Activity (U/ml)
300
200
100
0
0.20
0.15
0.10
0.05
Absorbance (280 nm)
100
75
50
25
0
NaCl (mM)
0
20
40
60
Fraction No. (2.2 ml)

Homogeneity, M_r, and pI of the Enzyme-Pig liver thioltransferase was demonstrated to be homogeneous by SDS polyacrylamide gel electrophoresis (Fig. 4), isoelectric focusing gel (Fig. 5), and high performance liquid chromatography of the reduced enzyme on a C18 reverse phase column (Fig. 6). The enzyme had a molecular weight of about 11,000 which is similar to rat liver thioltransferase and calf thymus glutaredoxin (9,16). Reduced enzyme had a pI of pH 6.4 while the disulfide treated enzyme had a basic pI of pH 8.0. The native enzyme also showed one pI species as reduced one (Fig. 5, lane D). But a minor band can appear at the position around the band of disulfide treated enzyme during freezing and thawing native enzyme.

Amino Acid Composition-The amino acid composition shown in Table 2 was obtained after 24 hour hydrolysis. The total cysteine content was determined by carboxymethylation of the denatured protein with iodoacetic acid in the presence of dithiothreitol. Tryptophan was not determined. Pig liver thioltransferase showed a very similar amino acid composition to that of rat liver thioltransferase and calf thymus glutaredoxin (Table 2).

N-Terminus Determination-After 5 cycles of Edman degradation of the carboxymethylated enzyme, no PTH derivative was detected, indicating the N-terminus was blocked.

Active Center Studies-The reduced enzyme was incubated

Fig 4. SDS polyacrylamide gel electrophoresis of purified pig liver thioltransferase. Lane A: Bio-Rad standard proteins (from top to bottom: phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa). Lane B: 5 ug pig liver thioltransferase. For details, see experimental procedures.

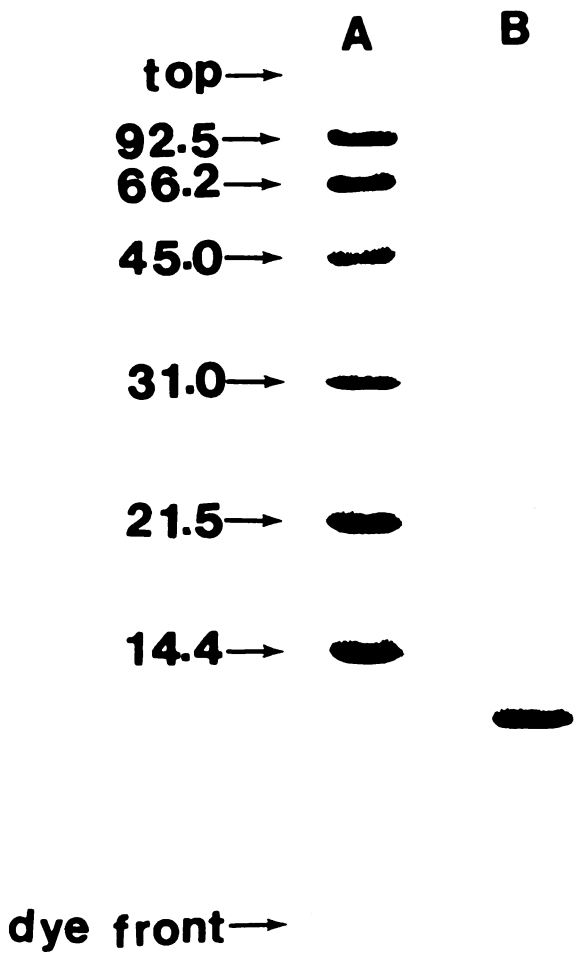

A
B
top
92.5
66.2
45.0
31.0
21.5
14.4
dye front

Fig 5. Isoelectric focusing of pig liver thioltransferase. Lane A: BDH pI protein markers. Lane B: 5 ug pig liver thioltransferase in 3 mM dithiothreitol. Lane C: 5 ug pig liver thioltransferase in 3 mM hydroxyethyl disulfide. Lane D: 5 ug pig liver thioltransferase from the second CM-Sepharose column. For details, see experimental procedures.

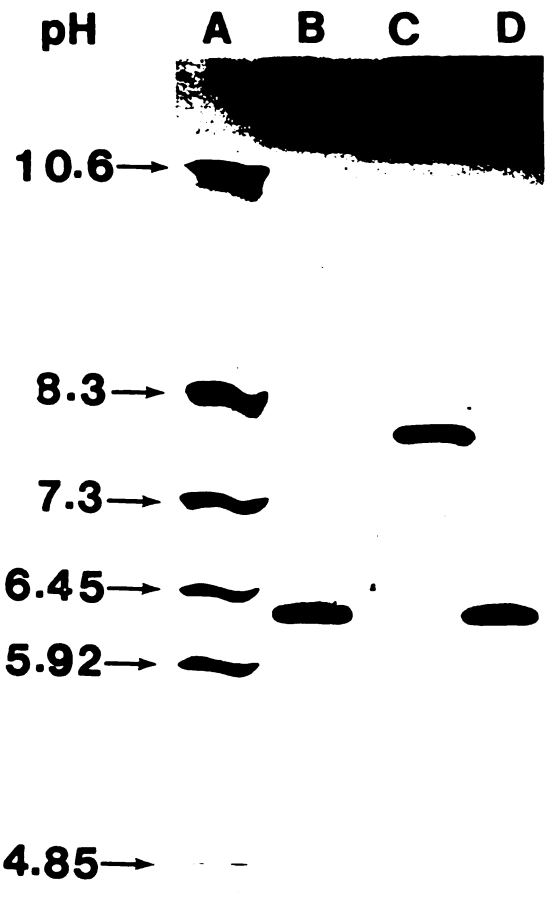

pH
A
B
C
D
10.6
8.3
7.3
6.45
5.92
4.85

Fig 6. High performance liquid chromatography of pig liver thioltransferase. A: 100 ug pig liver thioltransferase in 2 mM dithiothreitol. B: 2 mM dithiothreitol. A linear gradient of acetonitrile from 0 to 80% in 0.1% trifluoroacetic acid was generated during an 80 min period.

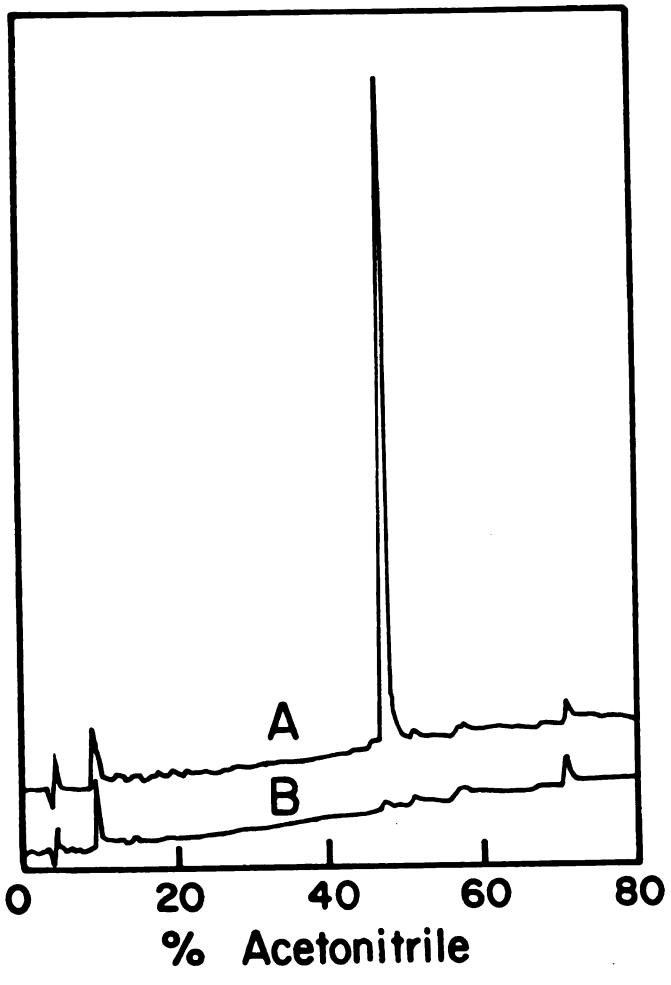

A
B
0
20
40
60
80
% Acetonitrile

TABLE 2

Amino acid composition of pig liver thioltransferase and comparison with rat liver thioltransferase and calf thymus glutaredoxin

	Amino acid composition		
	Pig liver thioltransferase[1]	Rat liver thioltransferase[2]	Calf thymus glutaredoxin[3]
Cysteine[4]	3.7(4)	4-5	4
Aspartic acid	5.2(5)	7	6
Threonine	8.8(9)	7	6
Serine	3.9(4)	4	3
Glutamic acid	17.0(17)	14	17
Proline	5.7(6)	4	4
Glycine	8.5(9)	7	9
Alanine	5.8(6)	5	6
Valine	5.2(5)	5	7
Methionine	1.0(1)	1	2
Isoleucine	6.2(6)	7	7
Leucine	12.5(13)	10	12
Tyrosine	0.8(1)	2	2
Phenylalanine	5.4(5)	5	5
Histidine	1.0(1)	0-1	1
Lysine	8.0(8)	7	6
Arginine	5.4(5)	4	4
Tryptophan	ND[5]	ND	ND
Total	(105)	94	101

1 Calculations were based on a protein of 11,500 Da and the numbers in parentheses were assumed integral numbers.
2 Gan, et al. (9). 3 Klintrot, et al.(20)
4 Determined as S-carboxymethyl cysteine following alkylation in the presence of dithiothreitol.
5 ND, not determined.

with iodoacetic acid at pH 6.0 and thioltransferase activity was determined at various times. About 90% of the thioltransferase activity was inhibited by 40 uM iodoacetic acid in 30 min (Fig. 7). However, when the enzyme was preincubated with S-sulfocysteine, the enzyme was totally protected. The protection probably resulted from sulfhydryl group oxidization or mixed disulfide formation between the enzyme and S-sulfocysteine.

Optimum pH-Thioltransferase activity was measured over the pH range of 5.5 - 9.5 under standard conditions (Fig. 8). The enzyme activity has an optimum at approximately pH 9.0 which is similar to that of the rat liver enzyme (9). At an acidic pH, thioltransferase activity was essentially not detectable. Since it is thought that the cysteine residues of the enzyme are involved in the reaction, the optimum pH in the alkaline range may reflect participation of the thiolate anion in the enzyme-catalyzed reaction. At a higher alkaline pH, the nonenzymatic reaction is significantly rapid.

Kinetic Behaviors-The two substrates, disulfides and GSH, were used to carry out kinetic studies. The plots of thioltransferase activities as a function of S-sulfocysteine, 2-hydroxyethyl disulfide and GSH concentrations did not exhibit normal Michaelis Menten kinetics (Figs. 9 and 10). At high concentrations of either disulfide substrate, the enzyme activities were inhibited (Fig 9). The $K_{0.5}$ for S-sulfocysteine and hydroxyethyl disulfide were estimated to

Fig 7. Pig liver thioltransferase activity in the presence of iodoacetate. Reduced Thioltransferase which was preincubated with buffer alone (Δ), 2.5 mM S-sulfocysteine (□), or 0.5 mM GSH (○) at room temperature for 10 min was incubated with 40 uM iodoacetate at room temperature and thioltransferase activity was determined at various times. The preincubation mixture (80 ul) contained 100 mM sodium phosphate, pH 6.0, about 100 uM reduced enzyme, and 2.5 mM S-sulfocysteine or 0.5 mM GSH. After 10 min incubation at room temperature, 100 ul of water were added and an aliquot of 90 ul was taken to incubate with 10 ul of 0.4 mM iodoacetate at room temperature. At various times, an aliquot of 10 ul was diluted 25 fold and thioltransferase activity was immediately measured.

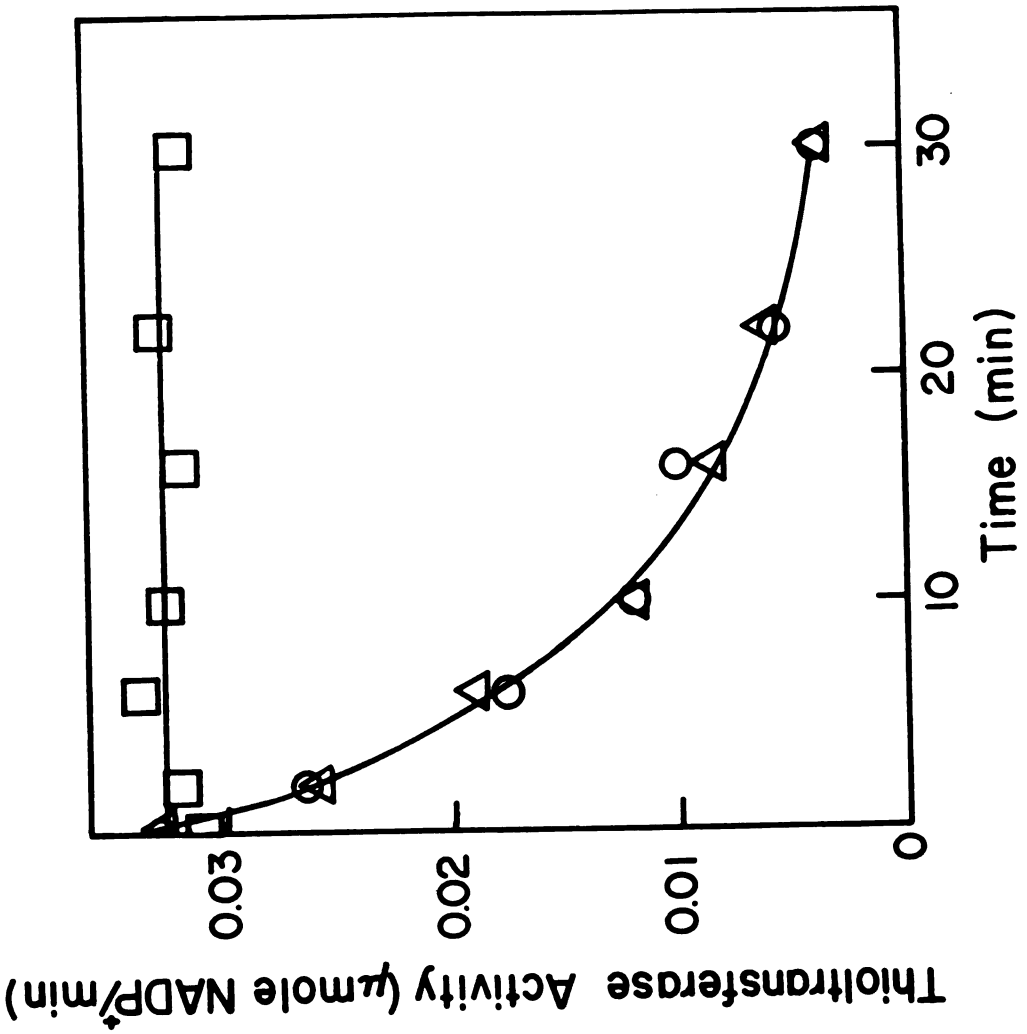

Thioltransferase Activity (μmole NADP/min)
0.03
0.02
0.01
0
10
20
30
Time (min)

Fig. 8. Pig liver thioltransferase activity dependence on pH. Thioltransferase 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 9.5) were used. Each assay contained 0.08 ug of purified pig liver thioltransferase. The net enzymatic (●) and spontaneous (Δ) velocities were described as the formation of micromoles of NADP/min.

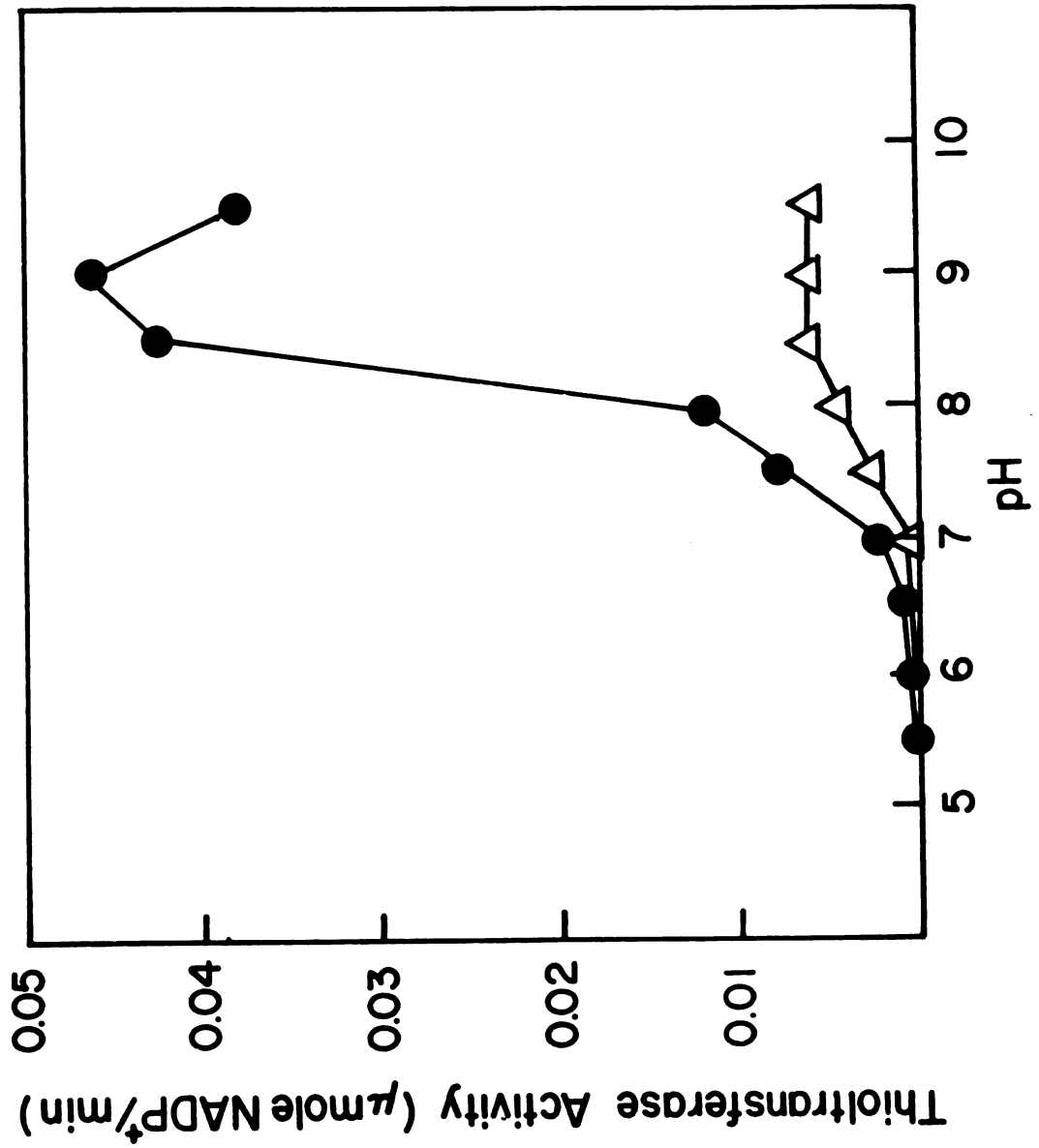

Thioltransferase Activity (μmole NADP+/min)
0.05
0.04
0.03
0.02
0.01
5
6
7
8
9
10
pH

Fig 9. Pig liver thioltransferase activity dependence on S-sulfocysteine and hydroxyethyl disulfide concentrations. The standard assay was used with increasing S-sulfocysteine (●) or hydroxyethyl disulfide (Δ) concentrations. Each assay contained 0.16 ug purified pig liver thioltransferase. Each value is the average of two separate experiments.

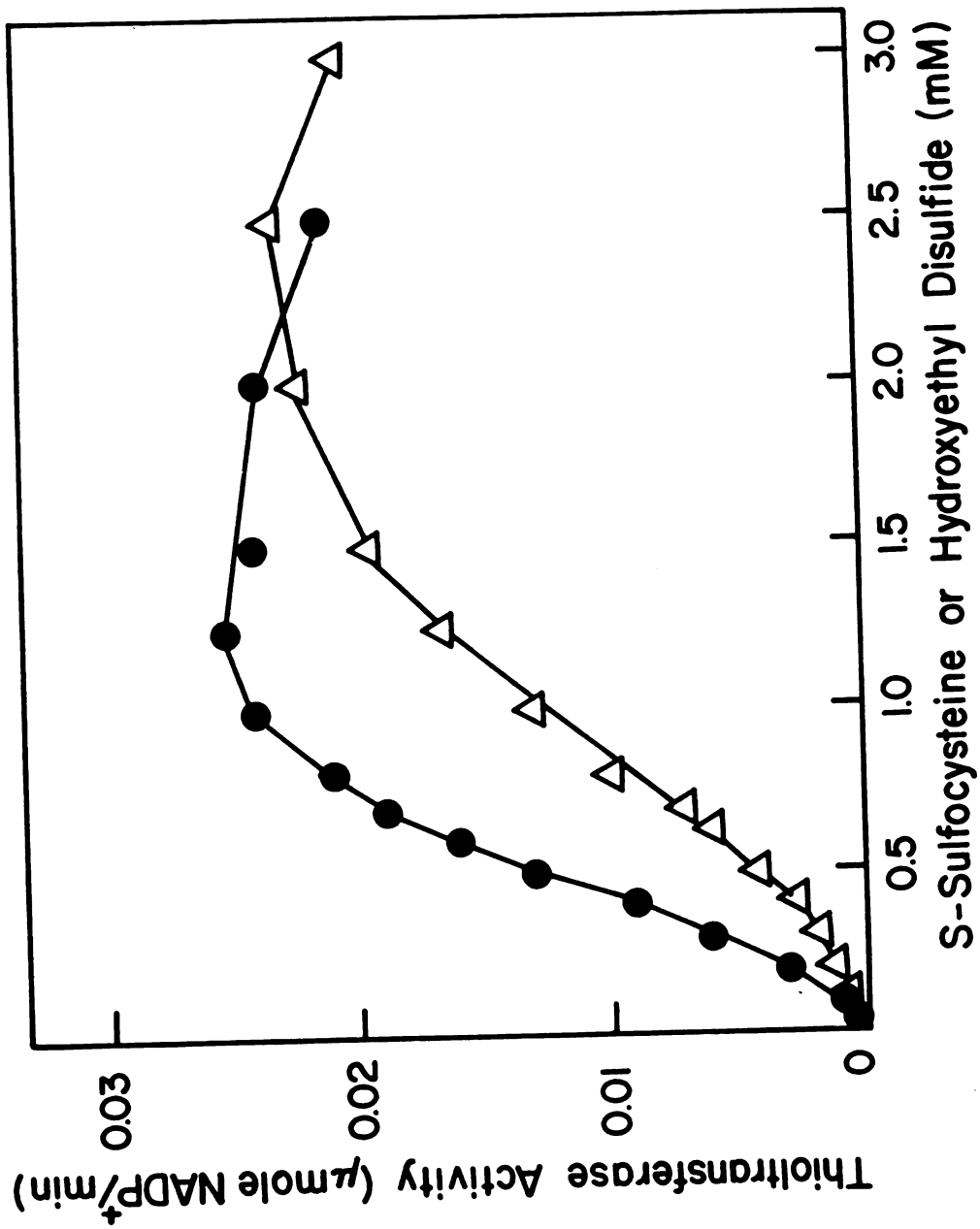
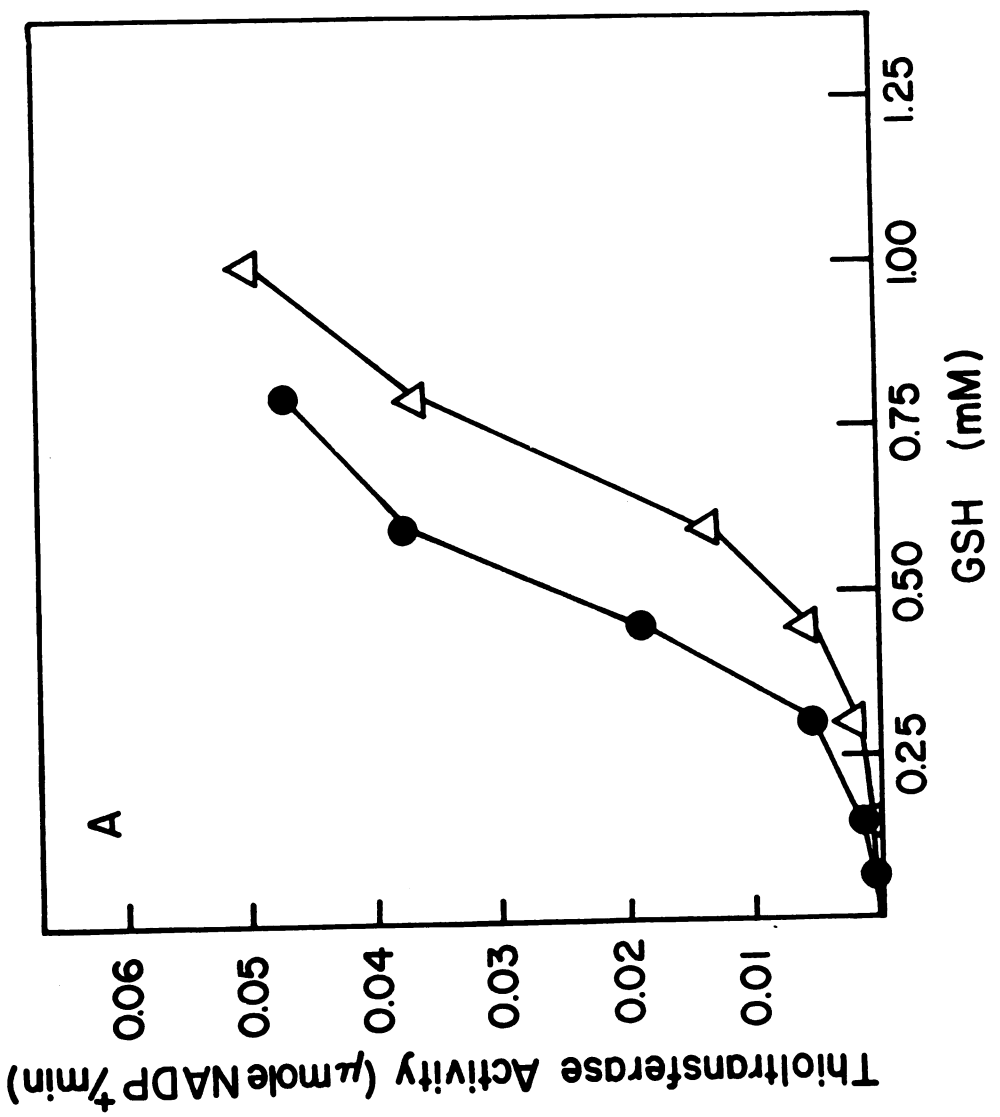
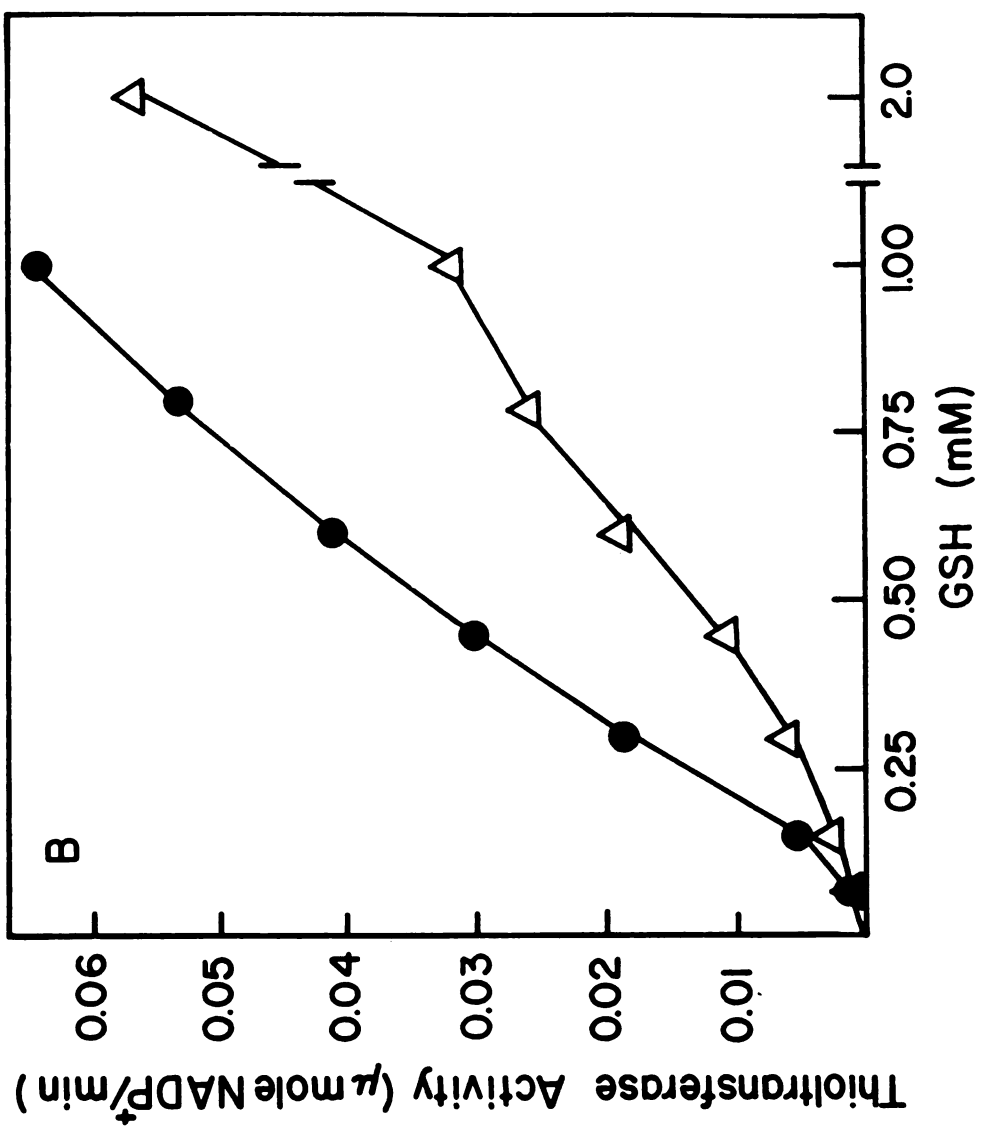

Thioltransferase Activity (μmole NADP+/min)
0.03
0.02
0.01
0
0.5
1.0
1.5
2.0
2.5
3.0
S-Sulfocysteine or Hydroxyethyl Disulfide (mM)

Fig 10. Pig liver thioltransferase activity dependence on GSH concentration. Different concentrations of GSH was used in the standard assay with constant S-sulfocysteine (A) or hydroxyethyl disulfide (B) concentrations. The solid circles were designated as net enzymatic reaction rate and the open triangles as spontaneous reaction rate. Each assay contained 0.12 ug purified pig liver thioltransferase. Each value is the average of two separate experiments.


A
0.06
0.05
0.04
0.03
0.02
0.01
Thiolitransferase Activity (μmole NADP+/min)
0.25
0.50
0.75
1.00
1.25
GSH (mM)


B
0.06
0.05
0.04
0.03
0.02
0.01
0.25
0.50
0.75
1.00
2.0
GSH (mM)
Thiolltransferase Activity (μ mole NADP+/min)

be 0.5 mM and 1.0 mM respectively. When GSH concentrations were increased, the spontaneous nonenzymatic reaction rates increased proportionally (Fig. 10). Since spontaneous reaction rates were too fast to be measured when GSH concentrations above 2 mM were analyzed in the presence of 2.5 mM S-sulfocysteine and 2-hydroxyethyl disulfide, no studies were carried out with greater than 2 mM GSH. At various GSH concentrations, hydroxyethyl disulfide gave lower spontaneous reaction rates as compared with S-sulfocysteine at pH 7.5.

DISCUSSION

The purification procedure described in this report provides an easy method for the isolation of relatively large quantities of homogeneous thioltransferase. From two kilograms of pig liver, I was able to obtain 24 mg of homogeneous enzyme. The prominent feature of this procedure was the deliberate reversible shift of the enzyme's pI thereby changing its elution position on the CM-Sepharose ion exchange column. Successful application of this strategy to pig liver thioltransferase permitted the purification of enough enzyme for future amino acid sequence analysis and the study of the reaction mechanism. The fact that the reduced enzyme has a lower pI than that treated with 2-hydroxyethyl disulfide implied that various sulfhydryl groups may be ionized at a physiological pH in the presence of dithiothreitol, whereas the same groups existed in intramolecular disulfide forms or in mixed disulfide forms when the enzyme was treated with hydroxyethyl disulfide. I have treated pure enzyme with hydroxyethyl disulfide at pH 5.5 (data not shown), and even at this pH, the enzyme was fully shifted to the oxidized form with a pI of 8.0. This result suggests that the pK of such a sulfhydryl group is very low. The pK of the sulfhydryl group of cysteine is reported to be 8.5 + 0.5 (17). However, Kallis and Holmgren

have studied the pK of the thiols of cysteine 32 and 35 at the active site of thioredoxin by means of the reaction of these groups with iodoacetic acid at various hydrogen ion concentrations (18). Cysteine-32 gave a pK of 6.7 whereas that of cysteine-35 was close to 9.0. It has been reported that thiol-disulfide interchange proceeds by way of a nucleophilic attack of a thiolate anion on the appropriate disulfide (19). Thus thioltransferase may also catalyze the reaction by a similar nucleophilic attack on disulfide substrates. Further studies on the kinetics of the reaction between thioltransferase cysteine residues and selected substrates may lead to new insights into the catalytic mechanism.

Often proteins that can not be purified to homogeneity by gel filtration and ion exchange chromatography alone may be purified by a suitable affinity chromatography column. However, a ligand with the proper affinity and specificity can not always be found. For enzyme proteins that are not irreversibly inhibited by the formation of either intramolecular or mixed disulfides, the possible shift in the pI resulting from oxidization or reduction of cysteine residues without denaturation may lead to a favorable shift in the elution position of the enzyme from an ion exchange chromatography support. I have no idea how many proteins may be successfully separated by this technique, but those that undergo a significant conformational shift during

thiol-disulfide exchange are likely candidates for an attendant protein surface pI shift. In addition to the successful purification of pig liver thioltransferase by this strategy, I have recently purified calf liver thioltransferase to homogeneity by the same technique (data not shown). In this case, the pI of the reduced form, 6.2, shifted to 7.7 after treatment of the enzyme with 2-hydroxyethyl disulfide.

The similarity between rat liver thioltransferase and calf thymus glutaredoxin has been previously discussed (8,9). In this report, we present data demonstrating the marked similarity between these proteins not only in molecular weight, amino acid composition, and disulfide reduction activity, but also in the blocked N-terminus which in the case of calf thymus glutaredoxin was reported to be a pyroglutamyl N-terminus by Klintrot et al. (20). Since calf thymus glutaredoxin has been sequenced (20), primary structure studies of pig and calf liver thioltransferase may provide more definitive information about these two glutathione-disulfide transhydrogenases.

ACKNOWLEDGMENTS

I wish to thank Dr. Young Moo Lee and Miss Doris Bauer for assistance in the amino acid composition analysis and N-terminus determination of pig thioltransferase.

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CHAPTER III

COMPLETE AMINO ACID SEQUENCE OF PIG LIVER THIOLTRANSFERASE

ABSTRACT

The complete amino acid sequence of pig liver thioltransferase has been determined. The homogeneous protein was cleaved by trypsin, chymotrypsin, Staphylococcus aureus V8 protease, and cyanogen bromide. The resulting peptides were purified by reversed phase HPLC and ion exchange FPLC. Sequencing of the fragments was achieved with either automated Edman degradation or Fast Atom Bombardment mass spectrometry. Pig liver thioltransferase is a single peptide with 105 amino acid residues and an acetylated glutamine N-terminus. The protein has two cysteine pairs with sequences of -Cys-Pro-Phe-Cys- and -Cys-Ile-Gly-Gly-Cys-, the first pair (Cys 22 and 25) being located at the potential active site of the enzyme. The sequence of pig liver thioltransferase displays close homology (82%) with calf thymus glutaredoxin, suggesting that they belong to the same evolutionary family.

INTRODUCTION

Thioltransferase has been found in yeast (1), rat liver (2,3), bovine liver (4), pig liver (5), and human placenta (6). There are numerous studies in the literature on its ability to transfer reducing equivalents from GSH to cytosolic protein and nonprotein disulfides and to regulate cytosolic enzyme activities (7-11). In a recent study, rabbit polymorphonuclear leukocytes, stimulated by immune complex, were found to release increased thioltransferase into the medium, in vitro, (12). In addition, these investigators reported that oxidized inactive papain was restored to 85% of original activity by treatment with both glutathione (GSH) and thioltransferase (12). Furthermore, a thioltransferase-like protein called soluble protein factor was found to promote the GSH-responsive iodothyronine 5' deiodinase activity in rat kidney microsomes (13-14). However, the complete physiological role of thioltransferase remains to be established.

Chemical characterization of thioltransferases from rat (15,16), bovine(4), and pig liver (5) has shown that they are small proteins with molecular weights of approximately 11,000. Each protein contains four cysteine residues and the enzyme is very sensitive to alkylating reagents (5,16). Carbohydrate (8.6%) has been found in homogeneous rat liver

thioltransferase (15). Further studies on the catalytic mechanism and physiological function of thioltransferase will require knowledge of the primary structure of the protein.

In this chapter, I describe the amino acid sequence of pig liver thioltransferase. This is the first description of the sequence of thioltransferase from any source, and comparison with the sequence of calf thymus glutaredoxin (82% homology) suggests that the two proteins may have identical cellular functions, though that possibility is not established.

EXPERIMENTAL PROCEDURES

Materials

Pig liver thioltransferase was prepared as previously described (5). TPCK treated trypsin and TLCK treated chymotrypsin were purchased from Sigma. Staphylococcus aureus V8 protease, trifluoroacetic acid, and guanidine hydrochloride were from Pierce. Cyanogen bromide was obtained from Aldrich. Iodoacetic acid (Sigma) was recrystallized from warm carbon tetrachloride. Acetonitrile (HPLC grade) was from J.T.Baker Chemical Co.. A reversed phase C18 HPLC column was purchased from Varian (Micro Pak, 0.4 x 30 cm). Mono Q HR 5/5 and Mono S HR 5/5 FPLC columns were from Pharmacia. All the other reagents were of either HPLC or analytical grade.

Methods

Carboxymethylation-Protein was carboxymethylated with iodoacetic acid as described by Gracy (17). Excess iodoacetic acid was removed by a Sephadex G-25 column.

Enzymatic Cleavage of Protein-All the proteolytic reactions were carried out in 0.2 M ammonium bicarbonate, pH 7.9, at 37° C. Tryptic cleavage proceeded for 6 hours at an

enzyme to substrate ratio of 1:75 (w/w). Chymotryptic digestion was carried out for 4 hours at an enzyme to substrate ratio of 1:60 (w/w). Cleavage with staphylococcus aureus V8 protease was carried out for 6 hours at an enzyme to substrate ratio of 1:60 (w/w). The buffer was removed by lyophilization.

CNBr Cleavage-Protein was first dissolved in 70% formic acid, and solid cyanogen bromide was added to give a concentration of 1.0 M. The reaction proceeded for 24 hours at room temperature. Excess cyanogen bromide was removed by a Speed Vac Concentrator.

Peptide Isolation-HPLC was performed on a Model 342 Beckman HPLC system equipped with an ISCO Model 1840 absorbance (214 nm) detector. All peptides were first purified by C18 reversed phase HPLC with acetonitrile gradients containing 0.1% trifluoroacetic acid. Some peaks were rechromatographed by HPLC in the presence of 25 mM ammonium acetate, pH 7.0. When peptides could not be purified by reversed phase HPLC, Mono Q or Mono S FPLC columns were employed.

Amino Acid Analysis-Peptides were hydrolyzed with constant boiling HCl in sealed evacuated reaction vials at 110° C for 24 hours. Amino acids were determined as phenylisothiocyanate(PITC) derivatives as described by Cohen (18).

Amino Acid Sequence Analysis-Sequence analysis was

performed by automated Edman degradation on a Beckman Model 890M sequencer. PTH derivatives were identified by two independent HPLC systems. The N-terminal peptide of the enzyme was determined by Fast Atom Bombardment (FAB) mass spectrometry. The FAB mass spectra were obtained with a Jeol JMS-HX110HF mass spectrometer and JeoL DA-5000 data system. The instrument was operated at resolution = 3000 and accelerating voltage 10kV. The fast atom beam was 6keV Xe atoms. The sample was dissolved in a glycerol matrix. Daughter scans were obtained by linked scanning at constant B/E using He as the collision gas.

RESULTS

Primary Structure-The amino acid sequence of pig liver thioltransferase together with the positions of peptides used to establish the sequence is shown in Fig. 11. The peptide fragments derived by standard enzymatic and chemical cleavage were first fractionated by reversed phase HPLC with increasing acetonitrile in the presence of 0.1% trifluoroacetic acid. Some peptides were rechromatographed either by reversed phase HPLC in the presence of 25 mM ammonium acetate, pH 7.0, or by anion exchange FPLC. The detailed results of peptide purification and amino acid compositions are given in the back of this Chapter (Fig. 14-17 and Table 4-7). The peptide sequences are consistent with their amino acid compositions measured. The amino acid sequence of pig liver thioltransferase is aligned with that of calf thymus glutaredoxin in Fig. 12. The degree of homology between these two proteins is 82%.

Trypsin Digestion and Mass Spectrometry-Since previous work showed that the N-terminus of pig liver thioltransferase was blocked (5), the enzyme was directly subjected to trypsin digestion. One of the tryptic peptides (T1) was resistant to Edman degradation. When this peptide was examined by Fast Atom Bombardment (FAB) (20) mass spectrometry, a protonated molecular ion, $(M + H)^+ = 906$, was observed. The daughter

Fig 11. The amino acid sequence of pig liver thioltransferase. The peptides derived from cleavage of pig liver thioltransferase with trypsin (T), chymotrypsin (C), Staphylococcus aureus V8 protease (V), and cyanogen bromide (CNBr) are shown. The peptide sequences obtained by both amino acid composition and sequence analysis are indicated by continuous solid lines, and those proven by only amino acid composition analysis by dashed lines. Ac in the N-terminus represents an acetyl group.

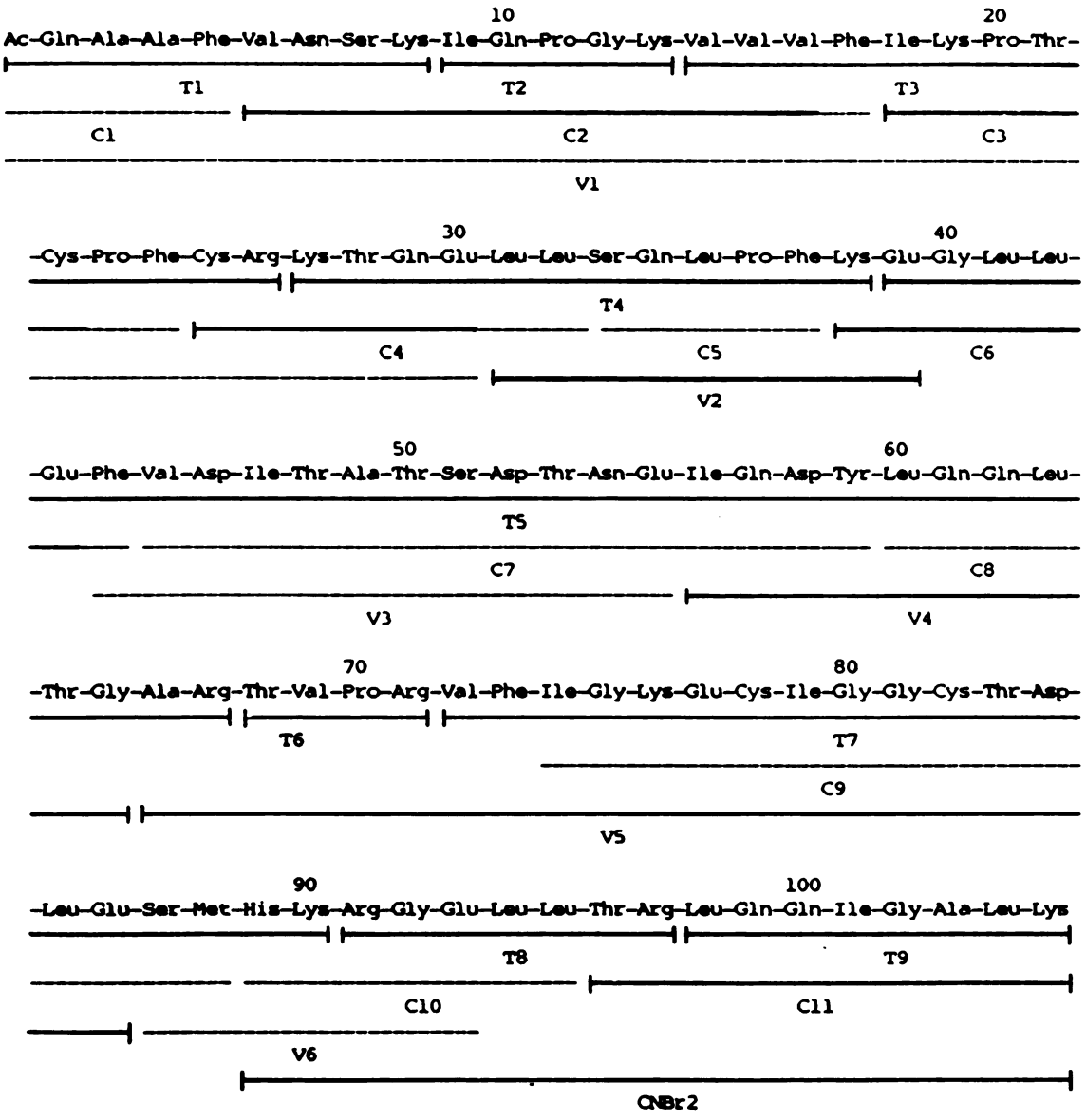
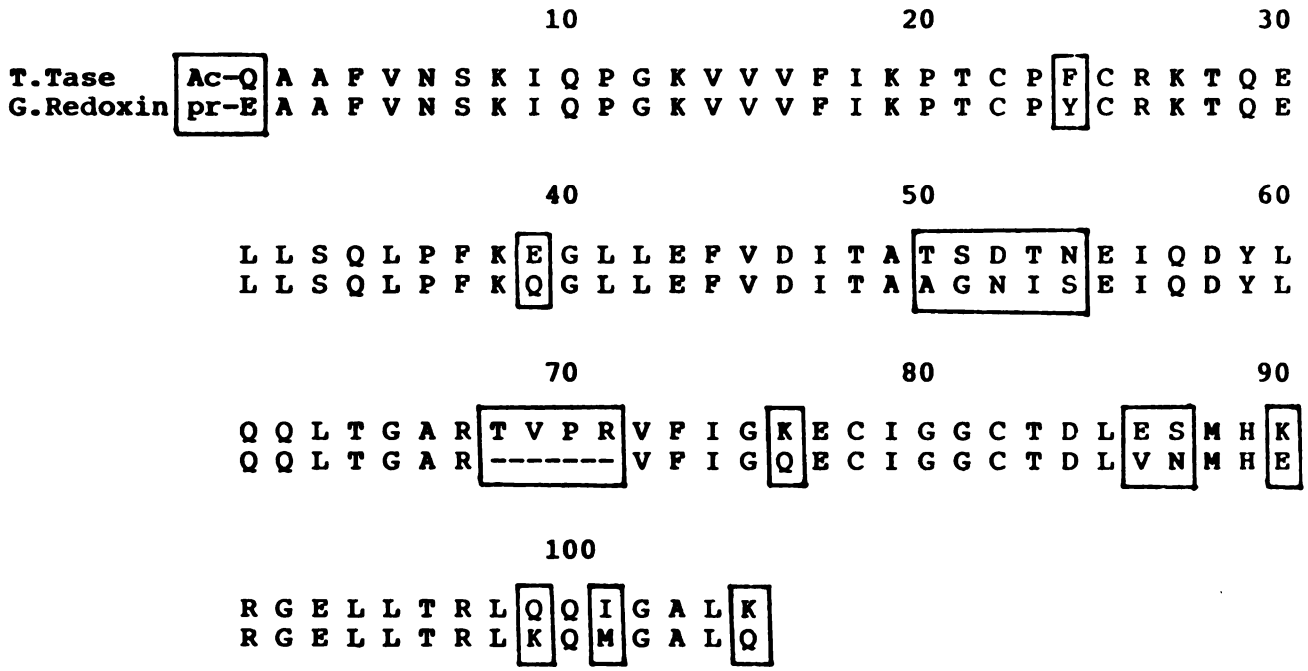

10
20
Ac-Gln-Ala-Ala-Phe-Val-Asn-Ser-Lys-Ile-Gln-Pro-Gly-Lys-Val-Val-Val-Phe-Ile-Lys-Pro-Thr-
T1
T2
T3
C1
C2
C3
V1
30
40
-Cys-Pro-Phe-Cys-Arg-Lys-Thr-Gln-Glu-Leu-Leu-Ser-Gln-Leu-Pro-Phe-Lys-Glu-Gly-Leu-Leu-
T4
C4
C5
C6
V2
50
60
-Glu-Phe-Val-Asp-Ile-Thr-Ala-Thr-Ser-Asp-Thr-Asn-Glu-Ile-Gln-Asp-Tyr-Leu-Gln-Gln-Leu-
T5
C7
C8
V3
V4
70
80
-Thr-Gly-Ala-Arg-Thr-Val-Pro-Arg-Val-Phe-Ile-Gly-Lys-Glu-Cys-Ile-Gly-Gly-Cys-Thr-Asp-
T6
T7
C9
V5
90
100
-Leu-Glu-Ser-Met-His-Lys-Arg-Gly-Glu-Leu-Leu-Thr-Arg-Leu-Gln-Gln-Ile-Gly-Ala-Leu-Lys
T8
T9
C10
C11
V6
CNBr2

Fig 12. Alignment of amino acid sequences of pig liver thioltransferase and calf thymus glutaredoxin. Amino acid sequences of pig liver thioltransferase and calf thymus glutaredoxin are given in the conventional single letter code. Ac represents an acetyl group. The N-terminus of calf thymus is a pyroglutamyl residue (19). A deletion is indicated by the dashed line. The residues in the amino acid sequences which differ from each other are boxed.


10 20 30
T.Tase Ac-Q A A F V N S K I Q P G K V V V F I K P T C P F C R K T Q E
G.Redoxin pr-E A A F V N S K I Q P G K V V V F I K P T C P Y C R K T Q E
40 50 60
L L S Q L P F K E G L L E F V D I T A T S D T N E I Q D Y L
L L S Q L P F K Q G L L E F V D I T A A G N I S E I Q D Y L
70 80 90
Q Q L T G A R T V P R V F I G K E C I G G C T D L E S M H K
Q Q L T G A R ------- V F I G Q E C I G G C T D L V N M H E
100
R G E L L T R L Q Q I G A L K
R G E L L T R L K Q M G A L Q

ion spectrum of m/z 906 (Fig. 13) showed fragment ions and the corresponding sequences (Table 3) which indicated that the N-terminal amino acid of the peptide was acetylated glutamine. The other major ions in Fig. 13 have been assigned to cleavage of the peptide backbone, or amino acid side chains. The FAB spectrum (not shown) also contained the B_1 and B_2 ions at m/z 171 and 242, respectively, confirming the N-terminal assignments. According to the exact mass (901.4666 measured vs. 901.4684 calculated), amino acid analysis, and the fragment ions, the only other alternatives for the N-terminus structure are some isomers of C_2H_4N-Glu. But none of these possibilities make biochemical sense leaving N-acetyl glutamine as the only reasonable choice.

Cyanogen Bromide Treatment-Since pig liver thioltransferase has one methionine, two peptides should be obtained from cyanogen bromide cleavage of the protein. Sequencing of the small peptide from the CNBr cleavage mixture (Fig. 15) gave 17 amino acid residues of the C-terminal peptide. However, no PTH derivative was found after 3 cycles of Edman degradation of the peptide representing the big peak of the CNBr peptides. SDS polyacrylamide gel electrophoresis of the CNBr cleavage mixture revealed the presence of two bands, one with the same M_r as the intact protein and the other with a M_r of 10,000 (data not shown), indicating that incomplete cleavage had occurred.

Chymotrypsin Digestion-It has been reported that

Fig 13. Fast Atom Bombardment CAD mass spectra of peptide T1 of pig liver thioltransferase. The molecular ions of peptide T1 underwent collisional activation dissociation (CAD) with He atoms. The spectrum above (daughter spectrum) was obtained by scanning the instrument at constant B/E. The values of B and Y" ions are given. All other peaks were attributed to cleavage of the peptide backbone or amino acid sidechains.

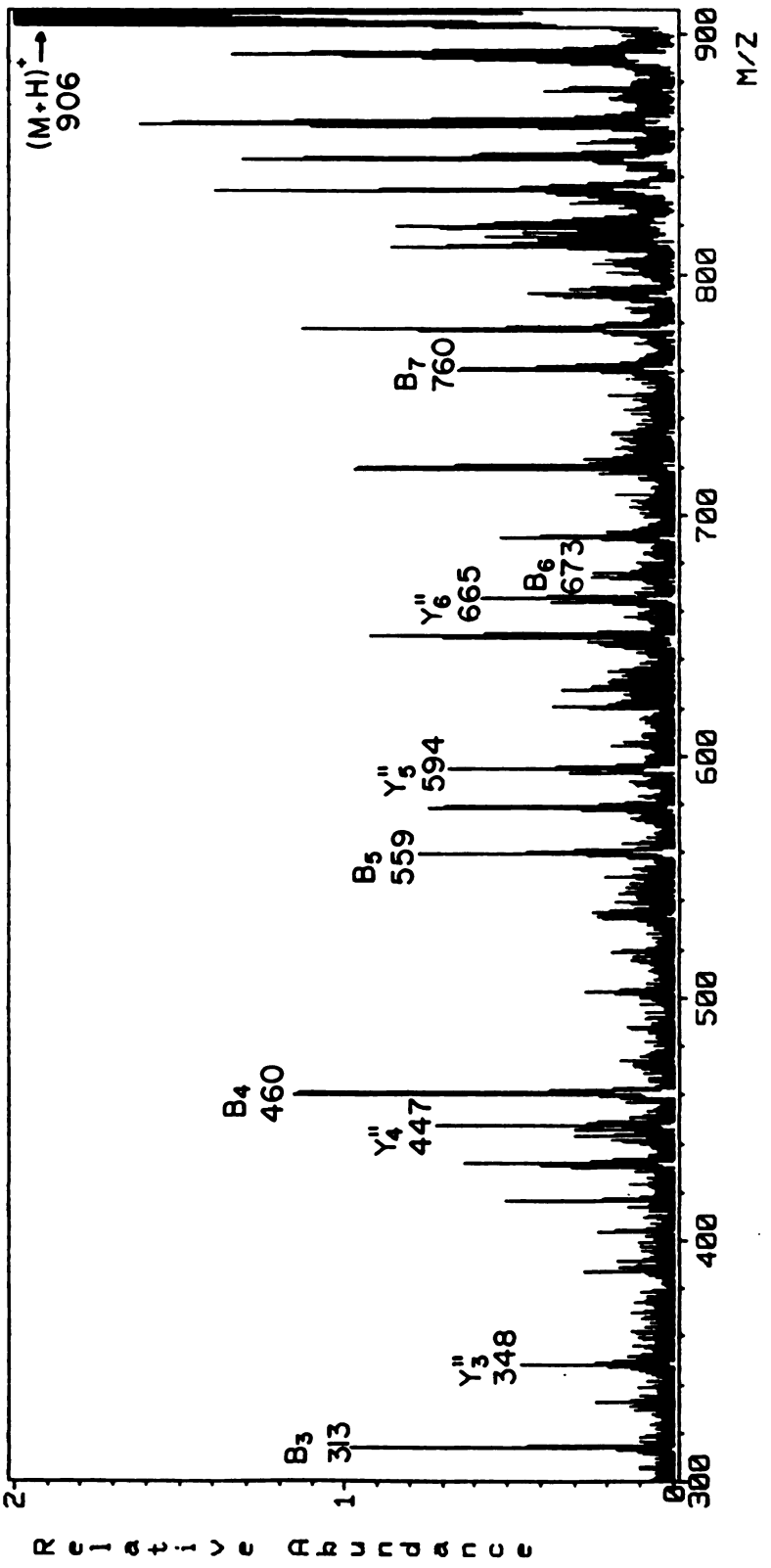

(M+H)+
906
B7
760
B6
673
Y"6
665
Y"5
594
B5
559
B4
460
Y"4
447
Y"3
348
B3
313
Relative Abundance
M/Z
2
1
0
300
400
500
600
700
800
900

TABLE 3

Fragment sequences of peptide T1 and their corresponding mass values observed in Fast Atom Bombardment CAD[1] spectra

Fragment Ion[2]	m/z	Amino acid sequence
B_3	313	Ac-Gln-Ala-Ala-
B_4	460	Ac-Gln-Ala-Ala-Phe-
B_5	559	Ac-Gln-Ala-Ala-Phe-Val-
B_6	673	Ac-Gln-Ala-Ala-Phe-Val-Asn-
B_7	760	Ac-Gln-Ala-Ala-Phe-Val-Asn-Ser-
$Y"_6$	665	Ala-Phe-Val-Asn-Ser-Lys-
$Y"_5$	594	Phe-Val-Asn-Ser-Lys
$Y"_4$	447	Val-Asn-Ser-Lys
$Y"_3$	348	Asn-Ser-Lys

1 Collisional activation dissociation.
2 The nomenclature for sequence of fragment ions in mass spectra follows the proposal of Roepstorff and Fohlman (21).

chymotrypsin may cleave peptides at the C-terminus of a methionine residue (22) in addition to the typical cleavage at amino acids with aromatic side chains. One such cleavage occurred at Met 88 of pig liver thioltransferase (Fig. 11). The best overlap information was provided by the sequences derived from chymotryptic and cyanogen bromide cleavage. During this study, we needed one more peptide to overlap the two gaps flanking the fragment T6. Sequencing one of the chymotryptic peaks with retention time of 28 min (Fig. 16), indicated that it contained the peptides Thr 64 to Phe 73 and Thr 68 to Phe 73. We tried to separate these two peptides by Mono Q and Mono S FPLC, but without success.

V8 Protease Digestion-To unambiguously establish the sequence of pig liver thioltransferase, the protein was digested with Staphylococcus aureus V8 protease. Peptide V5 derived from V8 protease cleavage contained the sequence which overlaps the trypsin peptides T5 to T7 (Fig. 11). However, the yield of the V8 protease digestion was low. The large peak seen at the high acetonitrile concentration region (Fig. 17) had the same retention time as the intact protein, suggesting incomplete digestion had occurred. Increasing the incubation time and enzyme/protein ratio did not appreciably improve the peptide yield from the V8 protease digestion. It is not clear why pig liver thioltransferase is resistant to Staphylococcus aureus V8 protease. An unusual cleavage by V8 protease, which normally prefers peptides adjacent to

glutamic acid residues, occurred at the peptide bond between Gly 65 and Ala 66. To confirm this kind of cleavage, both peptides V4 and V5 were sequenced, and the results agreed well with the reported cleavage (Fig. 11). To my knowledge, no such peptide preference has previously been reported for the V8 protease.

DISCUSSION

The high degree of homology between pig liver thioltransferase and calf thymus glutaredoxin supports the speculation that these two peptides, though isolated from different species, have the same function. It has been argued that thioltransferase from rat liver (15) and glutaredoxin from calf thymus (23), though similar in molecular weight, show significantly different catalytic properties (11). However, these kinetic differences may also be due to species variation. I have isolated a homogeneous protein from rat liver cytosol (16), calf liver cytosol (data not shown) and pig liver cytosol (5) which we labeled thioltransferase in accordance with the studies of Mannervik and his coworkers (3,8,15), but which have properties similar to the enzyme originally described by Racker (2) and designated glutathione:homocystine transhydrogenase. Glutaredoxin was originally discovered (24) in a mutant of E. coli lacking thioredoxin (25), but with a fully active NADPH-dependent ribonucleotide reductase function. Interestingly, a more convenient alternate assay for the measurement of glutaredoxin utilizes 2-hydroxyethyl disulfide, GSH, glutathione reductase and NADPH instead of the ribonucleotide reductase-coupled assay. The former assay and that using S-sulfocysteine as substrate are also commonly used in the

detection of thioltransferase activity (15, 16). Therefore, to resolve the question of identity, further studies must be conducted that include the assay of pig liver thioltransferase for ribonucleotide reductase activity and a thorough comparison of the kinetic properties of both thioltransferase and glutaredoxin isolated from the same species. Additional evidence can be obtained by immunochemical cross reactivity studies, but it would be surprising if peptides with 82% homology in primary structure did not react with antibodies raised against one another.

The N-terminal amino acid residue of pig liver thioltransferase is N-acetylated glutamine, whereas glutaredoxin from calf thymus has a pyroglutamyl N-terminus as determined by pyroglutamyl aminopeptidase and carboxypeptidase digestion (19). Acetylation of protein N-terminal amino acid groups has been found among a variety of structural proteins (26). Acetylation of a glutamine N-terminus is even more unusual as the great majority of proteins in this group possess a serine or an alanine at this position. N-acetylated thioltransferase is also not similar to other acetylated proteins which typically have charged residues in the N-terminal regions (26). The structural or functional significance of N-terminal acetylation is unknown. It appears to have no function in synthesis or folding of proteins. One possibility is to protect the protein against degradation by aminopeptidases or cathepsin C activity. This

speculation is in accord with the fact that a high proportion of acetylated proteins are structural in which stability towards degradation would seem essential. Thus, most viral capsid proteins, all major muscle proteins, some histones and several fibrous proteins are amino acetylated. One interesting analogy is found in the case of cytochrome c of mammalian origin (27), since this protein and thioltransferase have electron carrier functions, albeit with quite different chemical groups, and similar molecular weights.

Another apparent difference between thioltransferase and glutaredoxin is that pig liver thioltransferase has an extra tryptic peptide between positions 67 to 72. This tetrapeptide (-Thr-Val-Pro-Arg-) was not found in calf thymus glutaredoxin (19). Both proteins have two cysteine pairs. The cysteine 22 and 25 pair was proposed as the active center in calf thymus glutaredoxin (19). Pig liver thioltransferase has a similar sequence around this cysteine pair except that the calf thymus glutaredoxin Tyr 24 is replaced by a phenylalanine in pig liver thioltransferase. In addition, thioltransferase from rat liver cytoplasm has been reported to contain 8.6% carbohydrate (8), an unusual finding for a cytoplasmic protein. We have also observed the presence of PAS positive material within the band corresponding to homogeneous rat liver thioltransferase on SDS PAGE (16). On the other hand, whether glutaredoxin from mammalian sources

conta

glut

abil

whe

con

thi

stu

be

re

ce

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contains carbohydrate remains to be elucidated.

In summary, those investigators working with glutaredoxin have focused their studies primarily on its ability to function in the ribonucleotide reductase system, whereas those investigating thioltransferase have concentrated on a more general role in catalyzing cellular thiol-disulfide transhydrogenation reactions. Further studies are required to clarify the structural relationships between thioltransferase and glutaredoxin as well as the relative contribution of each, if they are not identical, to cellular functions involving the thiol/disulfide redox status.

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Fig 14. Reversed phase high performance liquid chromatography of tryptic peptides from pig liver thioltransferase. A C18 HPLC column was equilibrated with buffer A (0.1% trifluoroacetic acid), and a linear gradient of increasing acetonitrile was formed between 0 and 100% buffer B (70% acetonitrile in buffer A) with a flow rate of 1.0 ml per min. Peaks designated by a T, refer to tryptic peptides, and were subjected to amino acid composition and sequence analysis.

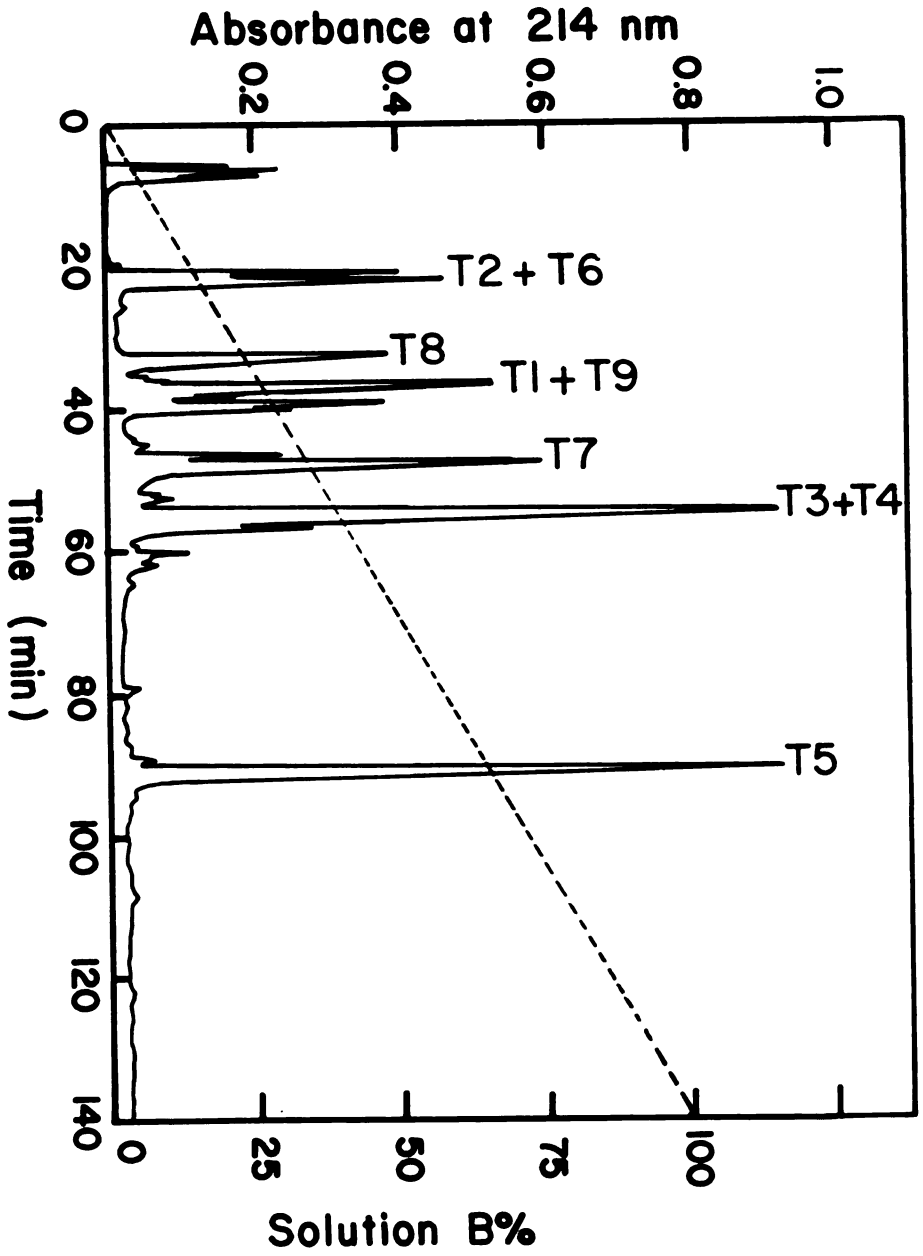

Absorbance at 214 nm
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
120
140
Time (min)
T2 + T6
T8
T1 + T9
T7
T3+T4
T5
0
25
50
75
100
Solution B%

Fig. 15. Reverse phase high performance liquid chromatography of cyanogen bromide peptides of pig liver thioltransferase. A C18 HPLC column was equilibrated with buffer A (0.1% trifluoroacetic acid), and a linear gradient of acetonitrile was formed between 0 and 100% buffer B (70% acetonitrile in buffer A) with a flow rate of 0.8 ml per min. Peaks designated by a CNBr 2, was subjected to amino acid composition and sequence analysis. The peak with a retention time of 48 min is a mixture of CNBr1 and intact protein as demonstrated by SDS polyacrylamide gel electrophoresis and N-terminal amino acid determination. For details, see text.

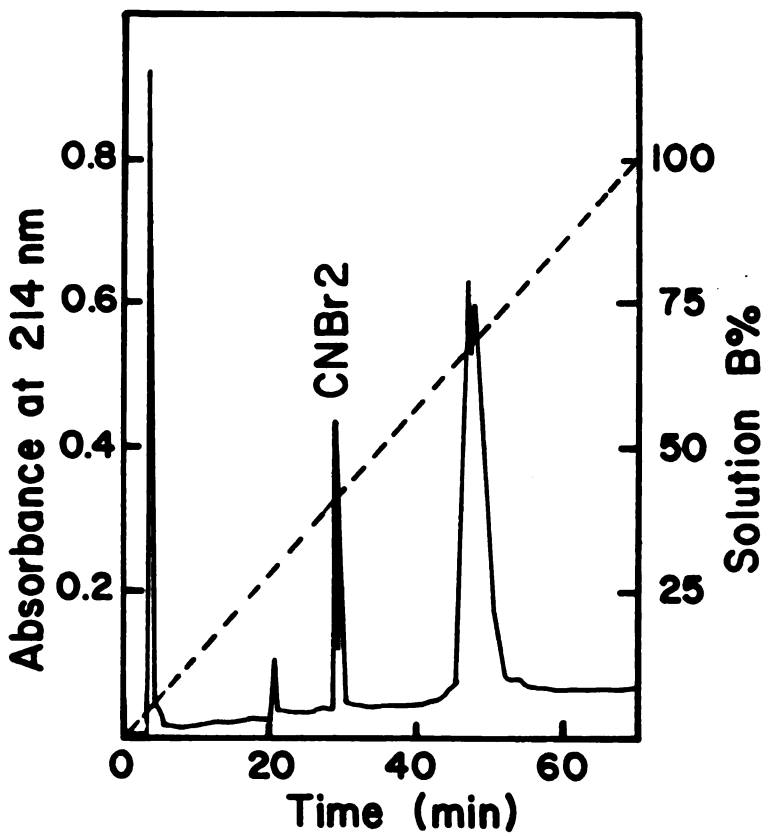

0.8
0.6
0.4
0.2
Absorbance at 214 nm
CNBr2
100
75
50
25
Solution B%
0
20
40
60
Time (min)

Fig 16. Reversed phase high performance liquid chromatography of chymotryptic peptides of pig liver thioltransferase. A C18 HPLC column was equilibrated with buffer A (0.1% trifluoroacetic acid), and a polyphasic gradient of acetonitrile was formed with buffer B (70% acetonitrile in buffer A) with a flow rate of 0.8 ml per min. Peaks designated by a C, refer to chymotryptic peptides which were subjected to amino acid composition and sequence analysis or only to amino acid determination. For details, see text.

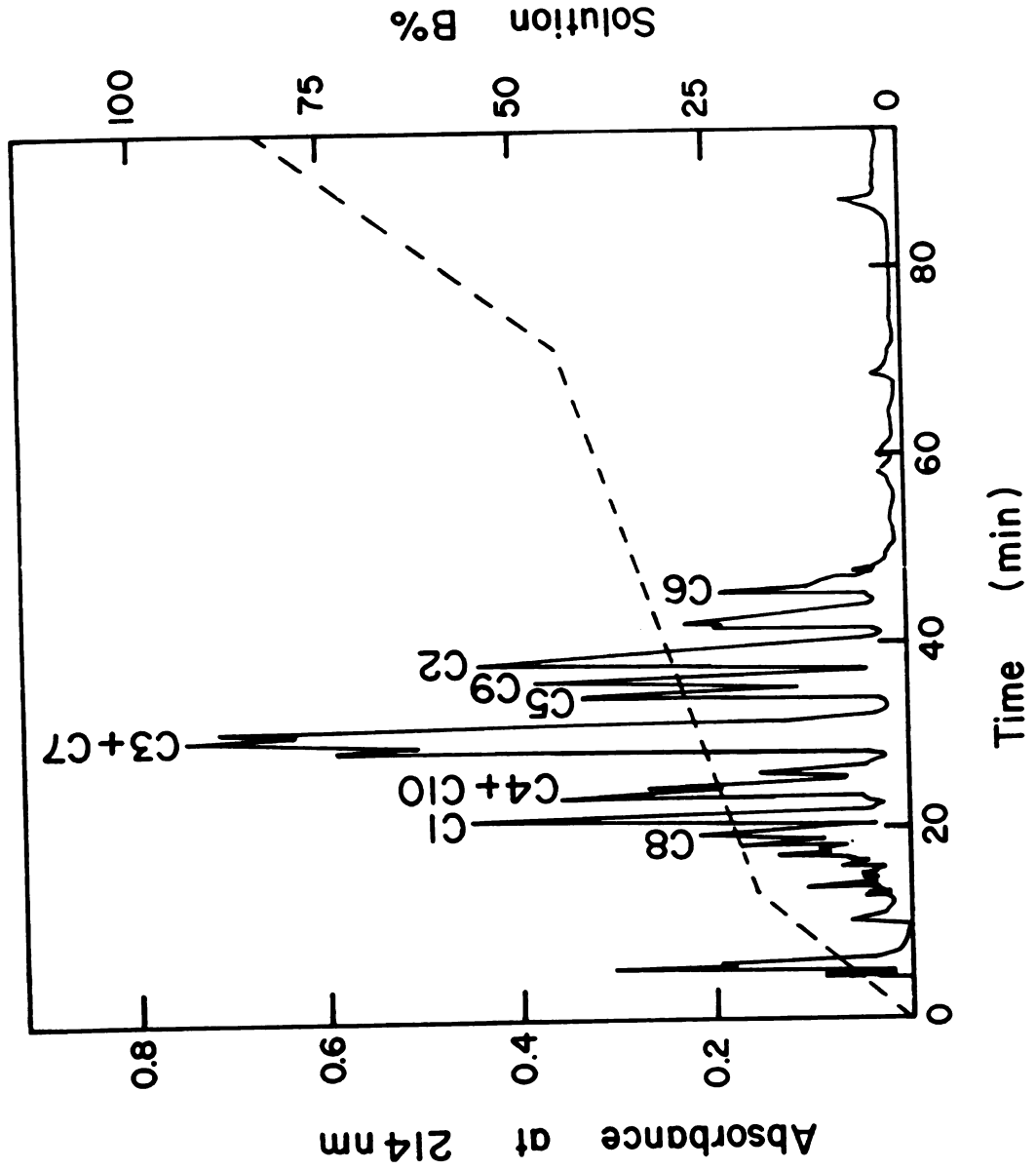

Absorbance at 214 nm
0.8
0.6
0.4
0.2
0
20
40
60
80
Time (min)
Solution B%
100
75
50
25
0
C8
C1
C4+C10
C3+C7
C5
C9
C2
C6

Fig 17. Reversed phase high performance liquid chromatography of Staphylococcus aureus V8 protease peptides of pig liver thioltransferase. A C18 HPLC column was equilibrated with buffer A (0.1% trifluoroacetic acid), and a linear gradient of acetonitrile was formed between 0-85% buffer B (70% acetonitrile in buffer A) with a flow rate of 0.8 ml per min. Peaks designated by a V, refer to Staphylococcus aureus V8 protease peptides which were subjected to amino acid composition and sequence analysis or only to amino acid composition determination. For details, see text.

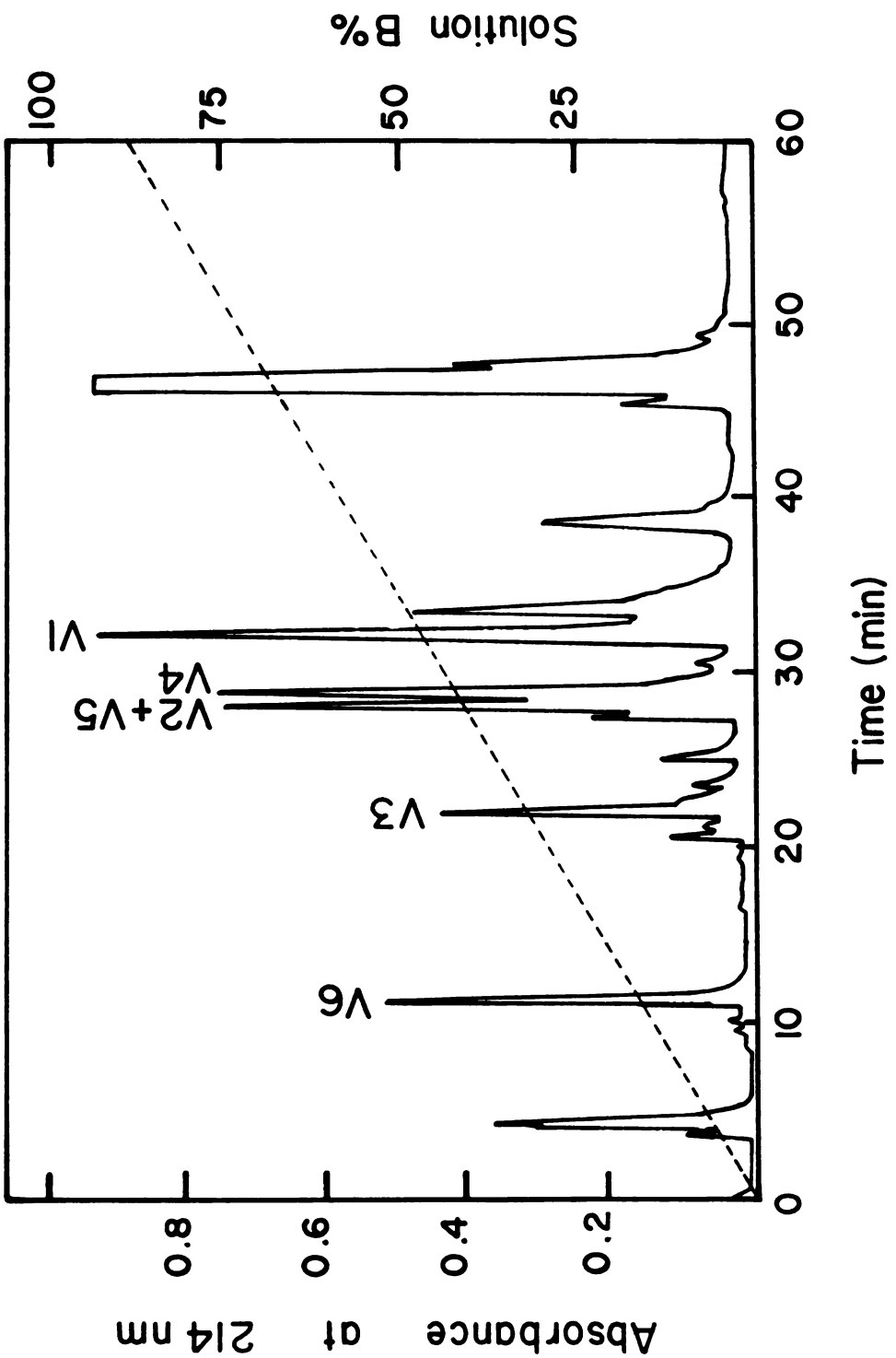

Absorbance at 214 nm
0.2
0.4
0.6
0.8
0
10
20
30
40
50
60
Time (min)
Solution B%
25
50
75
100
V6
V3
V2+V5
V4
V1

TABLE 4

Amino acid composition of the tryptic peptides
The values in parentheses are the numbers of amino acid residues determined by sequence analysis.

	T1	T2	T3	T4	T5	T6	T7	T8	T9
Cys(CM)			1.3(2)				1.4(2)		
Asx	0.6(1)				3.1(4)		0.6(1)		
Glx	1.0(1)	1.0(1)		2.1(3)	6.7(6)		1.9(2)	1.0(1)	1.8(2)
Ser	1.0(1)			0.7(1)	1.2(1)		1.0(1)		
Gly		1.2(1)			2.4(2)		3.0(3)	1.2(1)	1.0(1)
His							0.9(1)		
Arg			1.2(1)		1.2(1)	1.0(1)		2.0(2)	
Thr			1.0(1)	1.0(1)	4.2(4)	0.9(1)	1.0(1)	1.1(1)	
Ala	1.9(2)				2.1(2)				0.9(1)
Pro		1.1(1)	1.9(2)	1.0(1)		1.1(1)			
Tyr					0.8(1)				
Val	0.9(1)		2.4(3)		1.0(1)	1.2(1)	0.8(1)		
Met							0.8(1)		
Ile		I.0(1)	1.0(1)		2.0(2)		1.7(2)		0.9(1)
Leu				3.2(3)	4.0(4)		1.0(1)	2.0(2)	1.9(2)
Phe	0.9(1)		2.2(2)	1.1(1)	1.0(1)		0.7(1)		
Lys	1.0(1)	1.0(1)	1.2(1)	1.9(2)			2.0(2)		1.0(1)

TABLE 5

Amino acid composition of cyanogen bromide peptide CNBR2
The values in the parentheses are the numbers of amino acid residues determined by sequence.

Glx	2.2 (3)
Gly	2.3 (2)
His	1.0 (1)
Arg	1.4 (2)
Thr	1.2 (1)
Ala	1.4 (1)
Ile	1.1 (1)
Leu	4.5 (4)
Lys	2.0 (2)

TABLE 6

Amino acid composition of chymotrypsin peptides
The values in parentheses are the numbers of amino acid residues determined by sequence.

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
Cys(CM)			0.8(1)	0.6(1)					1.4(2)		
Asx		0.6(1)					3.5(4)		0.8(1)		
Glx	1.0(1)	1.0(1)		1.7(2)	0.9(1)	1.6(2)	2.5(2)	1.8(2)	1.7(2)	0.9(1)	1.8(2)
Ser		0.9(1)			0.8(1)		1.1(1)		0.9(1)		
Gly		1.1(1)				1.0(1)			2.9(3)	1.0(1)	1.0(1)
His										0.6(1)	
Arg				0.9(1)						1.0(1)	1.1(1)
Thr			0.8(1)	0.8(1)			2.9(3)		0.9(1)		0.8(1)
Ala	1.8(2)						1.0(1)				0.9(1)
Pro		1.5(1)	1.7(2)		1.0(1)						
Tyr							1.0(1)				
Val		3.7(4)					1.0(1)				
Met									0.6(1)		
Ile		1.1(1)	0.9(1)				2.6(2)		1.8(2)		1.0(1)
Leu				2.0(2)	0.9(1)	1.7(2)		2.0(2)	1.0(1)	2.0(2)	2.0(2)
Phe	1.0(1)	1.2(1)	1.0(1)		1.0(1)	1.0(1)					
Lys		2.5(2)	1.2(1)	1.1(1)		1.1(1)			1.2(1)	1.2(1)	1.2(1)

TABLE 7

Amino acid composition of staphylococcal V8 protease peptide. The values in parentheses are the numbers of amino acid residues determined by sequence

	V1	V2	V3	V4	V5	V6
Cys(CM)	1.7(2)				1.3(2)	
Asx	1.1(1)		3.1(3)	0.9(1)	1.2(1)	
Glx	3.6(4)	2.3(2)	2.0(1)	3.6(3)	1.6(2)	1.2(1)
Ser	1.0(1)	1.0(1)	0.9(1)			1.0(1)
Gly	1.4(1)			1.2(1)	3.5(3)	1.3(1)
His						1.0(1)
Arg	1.5(1)				2.8(2)	1.4(1)
Thr	2.4(2)		2.5(3)	1.0(1)	2.3(2)	
Ala	2.3(2)		1.1(1)		1.2(1)	
Pro	3.4(3)	1.0(1)			1.1(1)	
Tyr				0.7(1)		
Val	3.8(4)		1.0(1)		2.3(2)	
Met						0.8(1)
Ile	2.1(2)		0.9(1)	1.0(1)	2.0(2)	
Leu		2.8(3)		1.9(2)	1.0(1)	
Phe	2.7(3)	1.1(1)	1.0(1)		1.1(1)	
Lys	3.4(4)	1.0(1)			1.3(1)	1.0(1)

CHAPTER IV

IDENTIFICATION AND REACTIVITY OF THE CATALYTIC SITE OF PIG LIVER THIOLTRANSFERASE

ABSTRACT

The active site cysteine of pig liver thioltransferase was identified as Cys 22. The kinetics of the reactions of the Cys 22 with iodoacetic acid and iodoacetamide were studied. The rate constants of the reaction between Cys 22 and iodoacetamide as a function of pH revealed that the active site sulfhydryl group had a pKa of 3.8. Incubation of reduced enzyme with [1-14C]cystine prevented the inactivation of the enzyme by iodoacetic acid at pH 6.5 and no stable protein-cysteine disulfide was found when the enzyme was separated from excess [1-14C]cystine, suggesting an intramolecular disulfide formation. The kinetics of the reactions of reduced enzyme with S-sulfocysteine, one of the common substrates of the enzyme, or oxidized enzyme with reduced glutathione were monitored by inactivation with iodoacetic acid. The reaction rate between reduced enzyme and S-sulfocysteine was concentration dependent, but not pH dependent, while the reaction between oxidized enzyme and reduced glutathione was both concentration and pH dependent.

The results suggested a reaction mechanism for thioltransferase. The thiolate group of Cys 22 first initiates a nucleophilic attack on a disulfide substrate, resulting in the formation of an unstable mixed disulfide between Cys 22 and the substrate. Subsequently, the

sulfhydryl group at Cys 25 is deprotonated as a result of micro-environmental changes within the active site domain, releasing the mixed disulfide and forming an intramolecular disulfide bond. Reduced glutathione, the second substrate, reduces the intramolecular disulfide forming a transient mixed disulfide which is then further reduced by glutathione to regenerate the reduced enzyme and form oxidized glutathione. The rate limiting step for a typical reaction between a disulfide and reduced glutathione is proposed to be the reduction of the intramolecular disulfide form of the enzyme by reduced glutathione.

INTRODUCTION

Cytosolic thioltransferase functions as a thiol-disulfide oxidoreductase which catalyzes the reduction of low molecular weight disulfides and some protein disulfides by reduced glutathione (1-7). The reduced enzyme is very sensitive to alkylation reagents. However, preincubation of thioltransferase with its disulfide substrate can protect the enzyme from inactivation (8,9). Accordingly, it has been postulated that the active center of thioltransferase is cysteine dependent (8,9). A similar protein from E. coli thioredoxin, has two cysteine residues located near the N-terminus at positions -Cys^{32}-Gly-Pro-Cys^{35}- which participate in the active site of the enzyme (10). Furthermore, kinetic studies of the reactivity of the sulfhydryl groups toward iodoacetic acid and iodoacetamide as a function of pH revealed that the pK_a of Cys 32 was 6.7, whereas that of Cys 35 was 9.0 (11). In previous work (12), I have shown that pig liver thioltransferase contains two cysteine pairs with sequences of -Cys^{22}-Pro-Phe-Cys^{25}- and -Cys^{78}-Ile-Gly-Gly-Cys^{82}-, respectively. However, it has not yet been established which of these dithiol pairs are involved in the catalytic events.

In this chapter, I report that the active center of pig liver thioltransferase is located at cysteine 22. The

sulfhydryl group of this cysteine has a pKa of 3.8. The two substrates in a typical reaction, a disulfide and reduced glutathione, are capable of reversibly changing the redox form of the enzyme with different kinetic properties. These observations may suggest a possible mechanism for thioltransferase catalysis.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]iodoacetic acid with a specific activity of 6.25 mCi/mmole was from ICN Radiobiochemicals. DL-[1-^{14}C]cystine with a specific activity of 74 mCi/mmole was from Research Products International, Corp. Reduced glutathione, dithiothreitol, L-cystine, iodoacetic acid, iodoacetamide, and glutathione reductase were purchased from Sigma. Sephadex G-25 and a Mono Q HR 5/5 FPLC column were from Pharmacia. A reversed phase C18 HPLC column (Micro Pak, 0.4 x 30 cm) was from Varian. S-sulfocysteine was prepared by the method of Segel et al. and the product was recrystallized three times from aqueous ethanol (13). All the other reagents are either HPLC or analytical grade.

Methods

Thioltransferase Activity Assay-The enzyme was assayed as described previously(8). Briefly, the reaction mixture contained 0.5 mM GSH, 1.2 units of glutathione reductase, 2.5 mM S-sulfocysteine, 0.35 mM NADPH, 0.137 M sodium phosphate buffer, pH 7.5, and the enzyme to be assayed. The reaction proceeded at 30^{o} C and thioltransferase activity was measured

spectrophotometrically at 340 nm.

Preparation of Reduced Pig Liver Thioltransferase-The homogeneous enzyme was purified by a method previously described (9). The reduced enzyme was obtained by incubation with 20 mM dithiothreitol for 20 min at room temperature in 0.1 M sodium phosphate buffer, pH 7.0. Subsequent desalting was performed on a Sephadex G-25 gel filtration column (1 x 45 cm) which was equilibrated and eluted with water. The protein was collected through a pipet tip into a test tube and concentrated by lyophilization. To protect the enzyme from oxidation, the following storage conditions were used. The enzyme was kept at a high concentration (13-20 mg per ml), aliquots of 0.1 ml of the reduced enzyme were stored in a freezer, and each aliquot was thawed only once. Under the conditions described above, 99% of the enzyme was typically found in the reduced form, as demonstrated by iodoacetic acid inactivation.

Oxidation of the Reduced Enzyme-The reduced enzyme was treated with [1-^{14}C]cystine at an enzyme concentration of 0.42 mM and a cystine concentration of 0.67 mM at room temperature for 5 min in the presence of 0.1 M sodium phosphate buffer, pH 6.5. The oxidized enzyme was separated from excess cystine on a Sephadex G-25 column (1 x 45 cm) which was equilibrated and eluted with water. The protein was lyophilized.

Carboxymethylation-Protein was carboxymethylated with

iodoacetic acid as described by Gracy (14). Excess iodoacetic acid was removed by Sephadex G-25 chromatography as described above.

Tryptic and Chymotryptic Cleavage of the Protein and peptide Purification-The protein cleavage and the peptide purification were carried out under the same conditions described in the Chapter III.

Sequencing of the Active Center Peptide-The methods applied in the sequencing of the peptide were described in the Chapter III.

Protein Assay-Protein was assayed by the method of Lowry et al. (15) with bovine serum albumin as a standard.

RESULTS

Identification of the Active Site Cysteine of Pig Liver Thioltransferase-Preliminary results have shown that more than 90% of thioltransferase activity was lost after 20 min incubation of 0.12 mM reduced enzyme with 0.24 mM iodoacetic acid at a pH of 6.5 and at room temperature (data not shown). To identify which amino acid residue(s) react(s) with iodoacetic acid, the reduced enzyme (0.12 mM) was incubated with [1-^{14}C]iodoacetic acid (0.24 mM) in the presence of 0.1 M sodium phosphate, pH 6.5, at room temperature. After 20 min incubation, cold iodoacetic acid was added to give a concentration of 5 mM, and the excess iodoacetic acid was removed on a Sephadex G-25 column. Subsequent carboxymethylation, tryptic digestion, and peptide purification were performed under the same condition used in the Chapter III. The results of radioactivity counting of the tryptic peptides showed that only peptide T3 was labeled by [1-^{14}C]iodoacetic acid with more than background radioactivity. Peptide T3 was then subjected to automated Edman degradation and the total radioactivity of each residue was counted. The results given in Table 8 indicated that incorporation of ^{14}C was sharply increased at residue 9 which corresponds to cysteine 22 of the protein. The total cpm of the residues recovered before residue 9 were

TABLE 8

Identification of the active site amino acid of pig liver thioltransferase

Peptide T3 (15 nmol) was subjected to automated Edman degradation. Each degradation product was transferred to a scintillation vial with methanol. Total cpm of each residue was counted.

Residue Number	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13
Amino acid	Val	Val	Val	Phe	Ile	Lys	Pro	Thr[1]	Cys	Pro	Phe	Cys	Arg
Yield(nmol)	9.3	9.2	8.7	11	6.4	7.9	2.1		1.0	0.8	0.7	0.4	0.2
cpm x 10^{-2} (Total)	2.3	2.3	2.2	2.1	2.5	2.2	2.6	1.8	401	189	78	14	8.5
cpm x 10^{-2} (per nmol)	0.2	0.2	0.3	0.2	0.4	0.3	1.2		410	236	111	35	42

[1]The yield was not determined.

at the background level. The radioactivity observed with the residues following residue 9 was the result of carry-over, a common phenomenon which occurs in automated Edman degradation of peptides. These results strongly suggest that the active site is located at cysteine 22 of the protein. To further confirm this conclusion, the [1-^{14}C] iodoacetic acid labeled enzyme was digested by chymotrypsin, and the peptides were separated as described (12). It was found that peptide C3, containing only one cysteine, Cys 22, and terminating at Phe 24 (12), was the only peptide labeled by [1-^{14}C]iodoacetic acid.

Kinetics of the Reaction Between Cys 22 and Iodoacetic Acid-Since alkylation of Cys 22 will destroy catalytic activity of the enzyme, thioltransferase activity measurements should be a function of the concentration of reduced enzyme in an alkylation reaction of the enzyme. If only Cys 22 reacts with iodoacetic acid at pH 6.5 as seen above, and equal concentrations of reduced thioltransferase and iodoacetic acid are used, the reaction should follow second order reaction kinetics described as follows:

$$k = \frac{1}{t\,([TT\text{-}0] - [TT\text{-}CM])}$$

where k is the apparent rate constant, t is the time, [TT-0] is the concentration of reduced enzyme at time 0, and [TT-CM] is the concentration of carboxymethylated enzyme at time t.

The term ([TT-O] - [TT-CM]) should be a function of the thioltransferase activity. Equal concentrations (0.12 mM) of reduced enzyme and iodoacetic acid were incubated at room temperature in the presence of 0.1 sodium citrate buffer, pH 6.0. A plot of 1/thioltransferase activity against time gave a straight line with a rate constant of 0.96 $mM^{-1}min^{-1}$ and a half time of 8.7 min (Fig. 18). Since it is known that thiols are alkylated in their thiolate form (16), the reaction rate of alkylation should be strongly pH dependent. The rate constants of the reaction between reduced enzyme and iodoacetic acid were determined over a pH range of 1.4 to 6.6. A plot of the apparent rate constant against pH is given in Fig. 19. The apparent rate constants between pH 5 and 6.6 were not pH dependent, whereas they increased between pH 5 and pH 3, and decreased below pH 3. The rate constant was near 0 at pH 1.4. Since the degree of hydrogen ion dissociation of a sulfhydryl group should decrease as the pH decreases, an increase of the k value at pH 5 to 3 is probably not due to the increasing protonation of the sulfhydryl group but instead to effects on the alkylating reagent. Since iodoacetic acid has a pKa of 3.12 (17), the degree of carboxyl protonation of the reagent will increase and this decrease in negative charge may facilitate the reactivity of iodoacetic acid with the sulfhydryl group of the enzyme. This is in accord with the finding of Kallis and Holmgren that the apparent rate constant of iodoacetamide

Fig. 18. Rate plot of the reaction between equal concentrations (0.12 mM) of reduced pig liver thioltransferase and iodoacetic acid. The reactions were carried out at room temperature in the presence of 0.1 M sodium citrate buffer, pH 6.0. Thioltransferase activity was determined at various times after at least a 1000 fold dilution. [TT-SH] represent the reduced enzyme concentration calculated from the remaining thioltransferase activity.

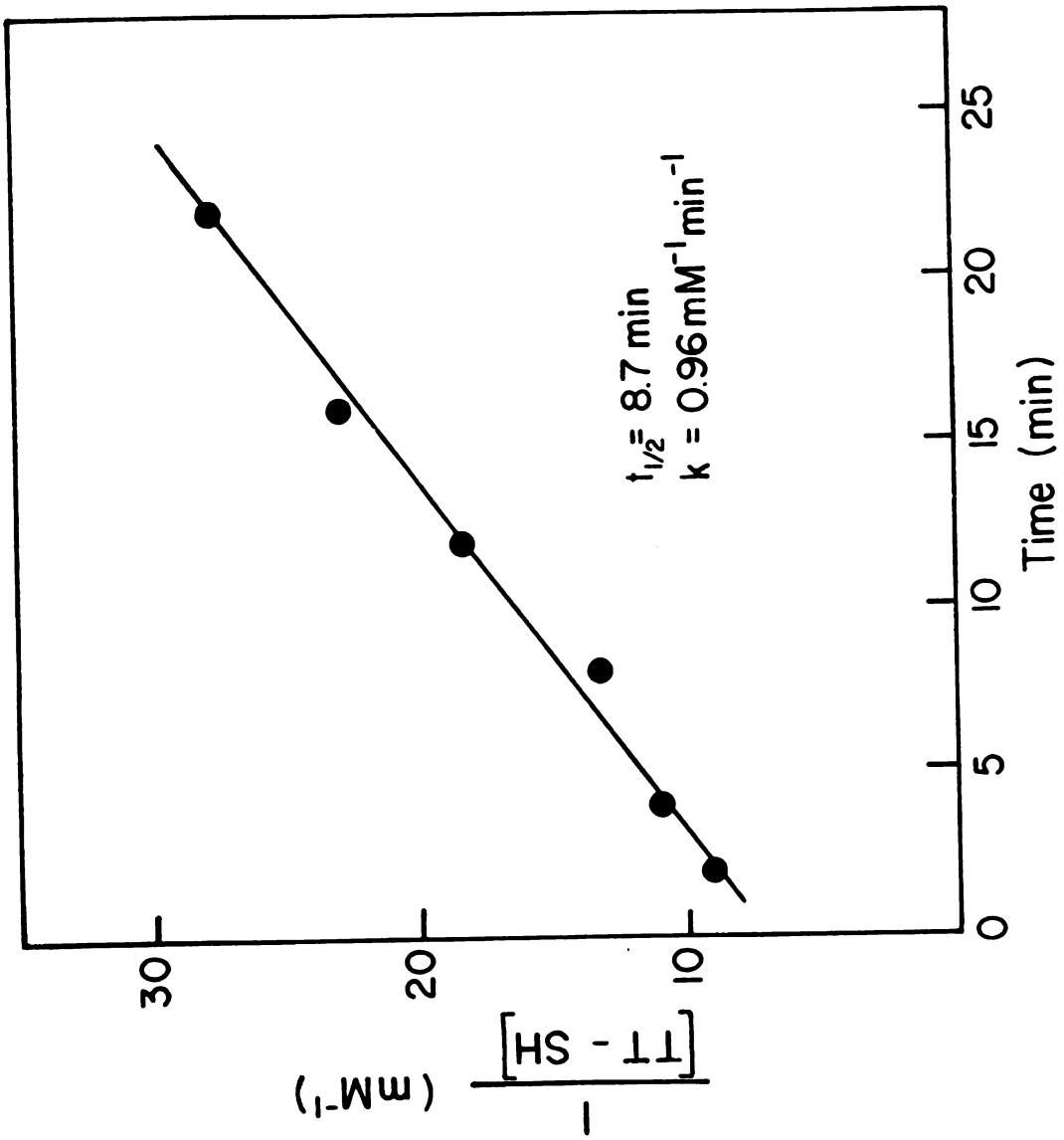
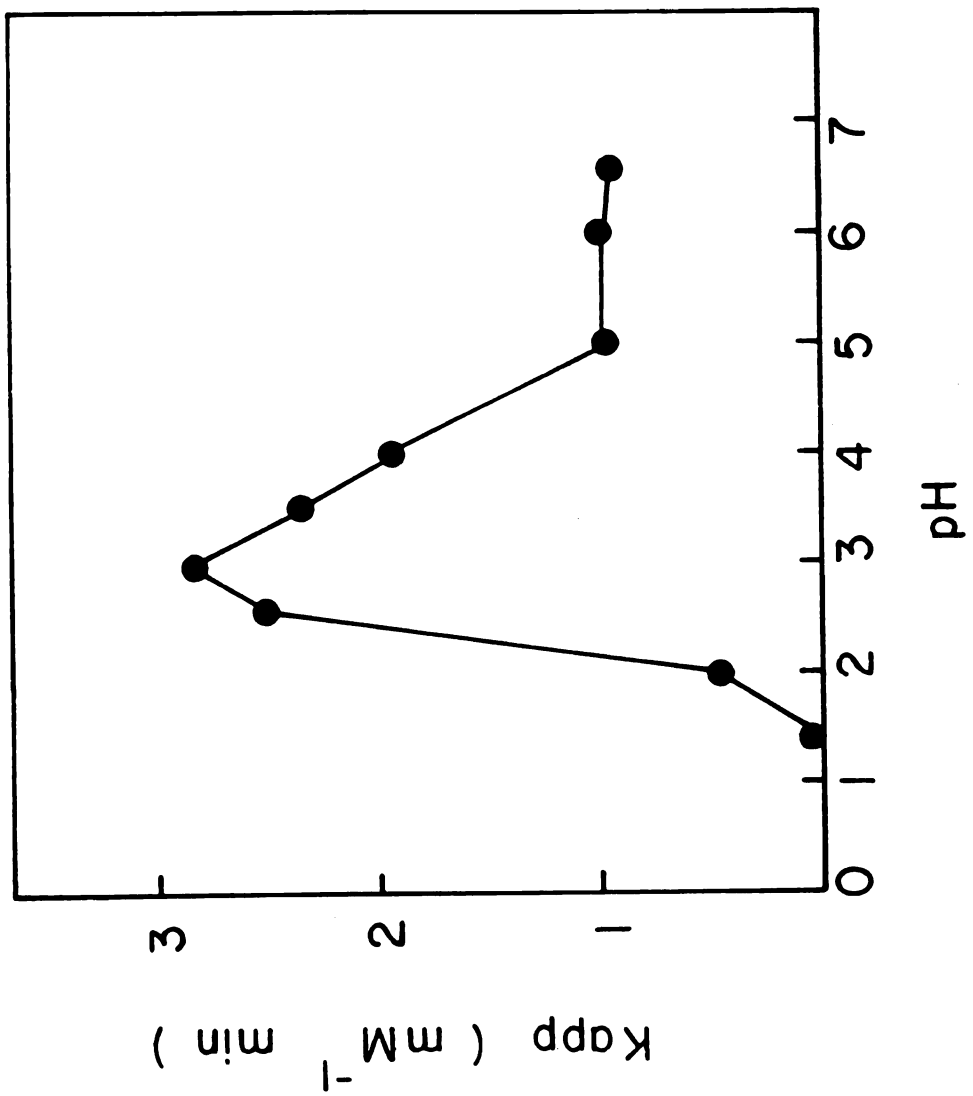

$\frac{1}{[TT - SH]}$ (mM^{-1})
30
20
10
0
5
10
15
20
25
Time (min)
$t_{1/2}$ = 8.7 min
k = 0.96 $mM^{-1}min^{-1}$

Fig. 19. pH dependence of second order apparent rate constants of the reactions between equal concentrations (0.12 mM) of reduced pig liver thioltransferase and iodoacetic acid. The k_{app} were determined by the methods described in Fig. 18. Blank thioltransferase activity was not effected when the pH decreased over the indicated range.


kapp (mM^{-1} min)
3
2
1
0
1
2
3
4
5
6
7
pH

with no negative charge was 20 fold higher than that of iodoacetic acid at pH 7.2 in similar studies with thioredoxin (11). However, it is possible that the low pH may cause a conformational change of the protein whereby iodoacetic acid could come into closer proximity to the sulfhydryl group, resulting in the increased reaction rate. The sharp decrease of the apparent rate constants below pH 3 may be caused by protonation of the active site sulfhydryl group. The interpretation of the kinetics above is based on the assumption that the alkylation reaction of the enzyme is a function of the catalytic activity, and only one sulfhydryl group of the enzyme reacts with iodoacetic acid under the conditions used. If this is true, the degree of iodoacetic acid incorporation into the enzyme should be proportional to the apparent rate constants at different pH values. To investigate this hypothesis further, reduced pig liver thioltransferase (0.12 mM) was alkylated by [1-14C]iodoacetic acid (0.15 mM) in the presence of 0.1 M sodium citrate buffer at different pH values. Excess cold iodoacetic acid was added to the mixture after 3 min incubation at room temperature, and the enzyme was separated from excess iodoacetic acid by a Sephadex G-25 column. The radioactivity incorporated into the enzyme is given in Table 9. The incorporation pattern is in good agreement with the apparent rate constant curve shown in Fig. 19. The protein labeled at different pH values was subjected to chymotryptic digestion

TABLE 9

Incorporation of [1-^{14}C]iodoacetic acid into pig liver thioltransferase at different pH

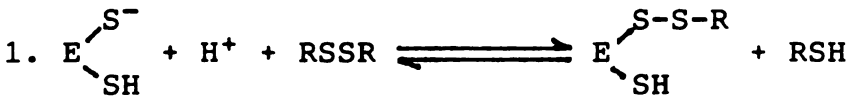
Reduced enzyme (0.12 mM) was incubated with [1-^{14}C]iodoacetic acid (0.15 mM) in 0.1 M sodium citrate buffer at different pH. After 3 min incubation at room temperature, excess cold iodoacetic acid was added to give a final concentration of 5 mM, and the mixtures were immediately loaded onto Sephadex G-25 columns to desalt. Total cpm and protein content of protein fractions of Sephadex G-25 chromatography were determined. The molecular weight of the enzyme used in the calculation of moles of carboxymethyl group incorporation/mole of enzyme was 11,500.

pH	1.4	2.0	3.0	4.0	5.0	6.0
Moles carboxymethyl groups incorporated per mole of enzyme	0.04	0.10	0.49	0.37	0.21	0.22

purified by reversed phase HPLC. It was found that only peptide C3 was labeled, suggesting that cysteine 22 was the only amino acid residue of the protein which reacted with iodoacetic acid under the conditions used.

pH Dependence of the Reaction between the Reduced Enzyme with Iodoactamide-Since the kinetics of the reaction between Cys-22 and iodoacetic acid were complicated by protonation of the carboxyl group of iodoacetic acid, I have determined the rate constants of the reaction with iodoacetamide at different pH values (Fig. 20). The apparent second order rate constant showed a pronounced pH dependence in the region around pH 3.8 whereas it was essentially constant between pH 4.9 and 6.6. The results suggest that the active site Cys-22 has a pKa of 3.8.

Disulfide Protection of the Enzyme from Iodoacetic acid Inactivation-Previous results showed that the disulfide substrates (RSSR) of the enzyme can protect the enzyme from inactivation by iodoacetic acid (8,9), but the mechanism was not clear. According to the results described above, i.e., only one sulfhydryl group reacted with iodoacetic acid, three mechanisms can be proposed:



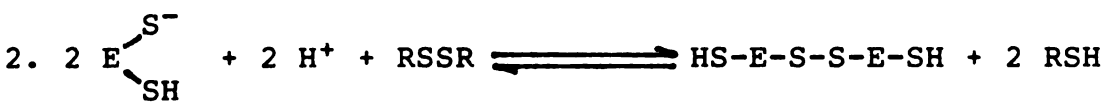
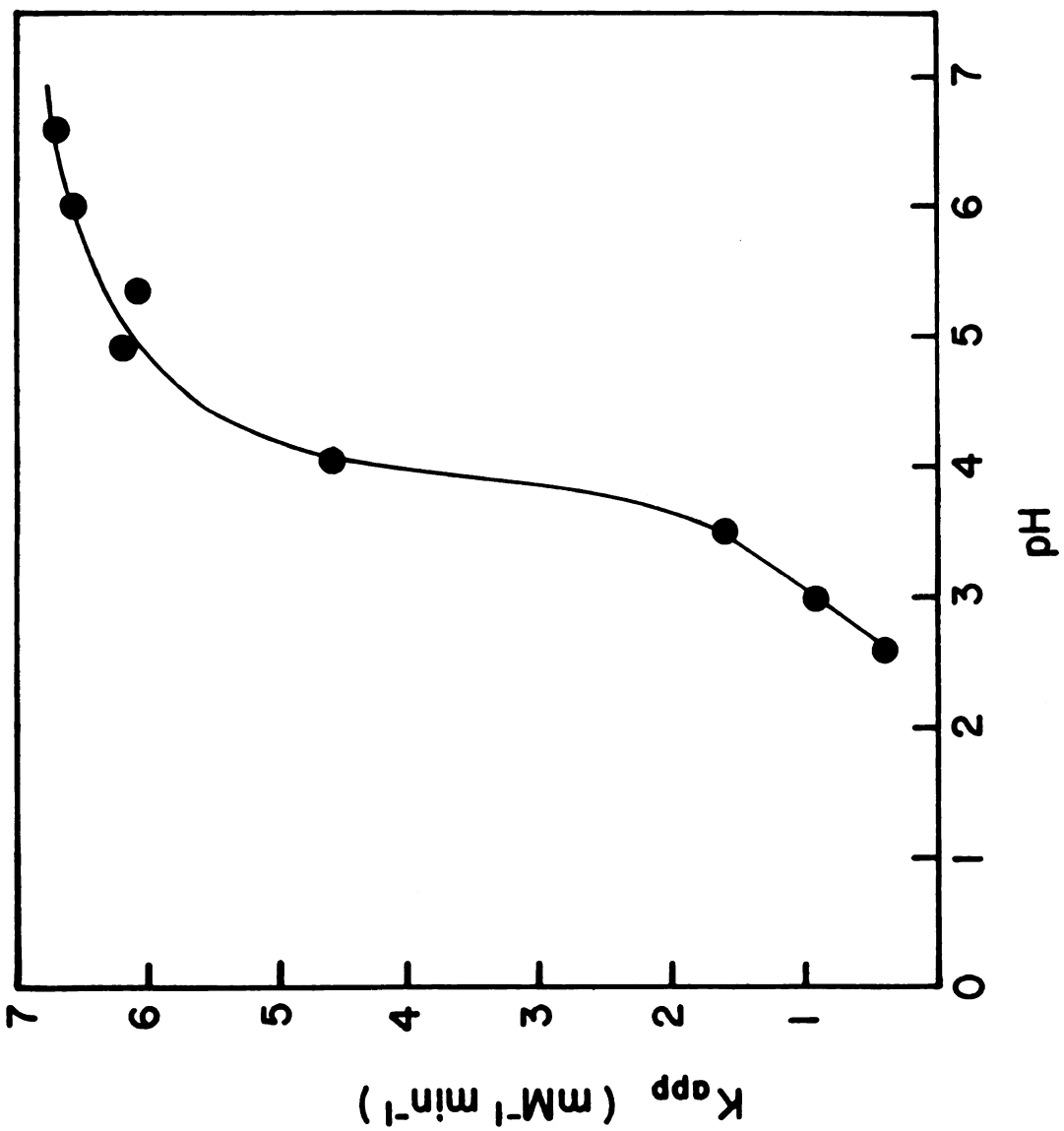
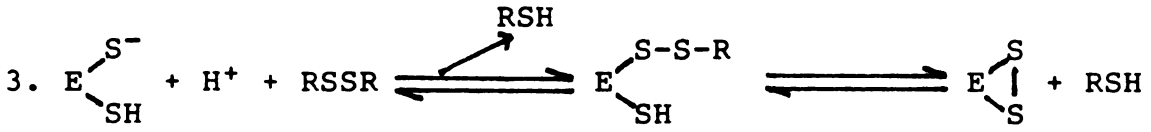


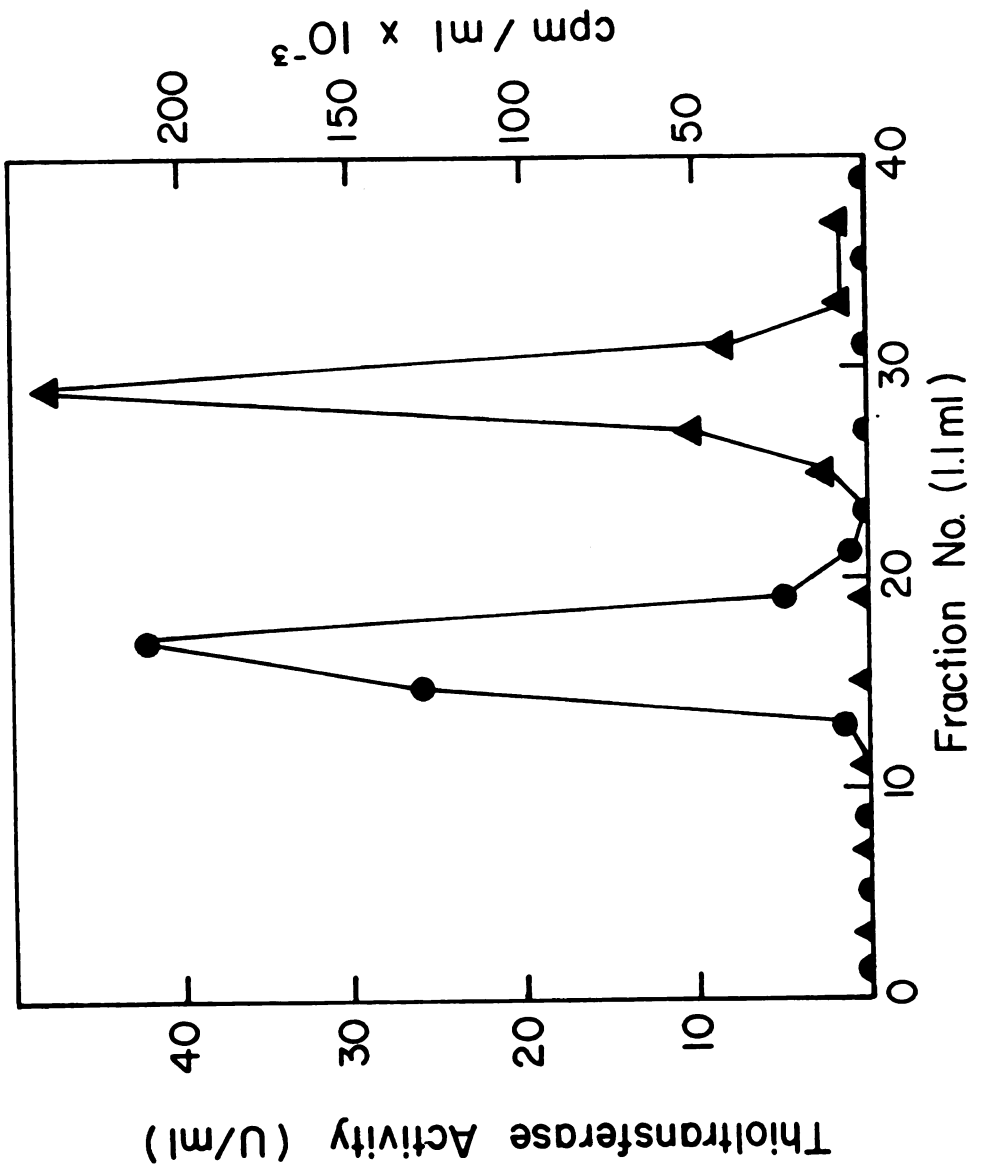
Fig. 20. pH dependence of second order apparent rate constants of the reactions between equal concentrations (0.06 mM) of reduced pig liver thioltransferase and iodoacetamide. The reactions were carried at room temperature in the presence of 0.1 M sodium citrate buffer from pH 2 to pH 6.6. The thioltransferase activity was determined at various times after a 800 dilution. The second order rate constants were obtained by plotting 1/[TT-SH] against times. [TT-SH] represents the reduced enzyme concentration calculated from the remaining thioltransferase activity.


K_{app} (mM^{-1} min^{-1})
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
pH



To test these mechanisms, reduced pig liver thioltransferase (0.42 mM) was incubated with [1-^{14}C]cystine (0.67 mM) at room temperature for 5 min in the presence of 0.1 M sodium phosphate buffer, pH 6.6. The subsequent Sephadex G-25 chromatography was performed to separated the enzyme from excess [1-^{14}C]cystine, and the thioltransferase activity and the radioactivity of the fractions were measured. Consistent with previous results (8,9), thioltransferase was totally inactivated by iodoacetic acid before incubation with [1-^{14}C]cystine, whereas the enzyme, preincubated with [1-^{14}C]cystine, retained its full enzymatic activity and insensitivity to iodoacetic acid treatment, but was unlabeled with ^{14}C (Fig. 21), ruling out mechanism number 1. Another possibility for the protection mechanism is the formation of a dimeric species of the enzyme (equation 2). In this mechanism, the enzyme would be protected against iodoacetic acid treatment and would not be radioactively labeled. However, when the [1-^{14}C]cystine treated enzyme was run on SDS polyacrylamide gel electrophoresis under nonreducing conditions in the presence of excess iodoacetic acid, no shift in the molecular weight of the enzyme on the gel (data not shown) was observed, ruling out mechanism 2. However, the results obtained in this study fully support the

Fig. 21. Interaction between [1-^{14}C]cystine and reduced pig liver thioltransferase. The solid circles and triangles represent thioltransferase activity and cpm of the fractions, respectively. For details, see Experimental Procedures.


Thioltransferase Activity (U/ml)
cpm / ml x 10^{-3}
Fraction No. (1.1 ml)
0
10
20
30
40
50
100
150
200

mechanism described in reaction 3, namely, the formation of an intramolecular disulfide bond.

Reactivity of the Enzyme with S-sulfocysteine and Reduced glutathione-Previous results have shown that the reaction between the reduced enzyme and S-sulfocysteine was completely blocked by an excess of iodoacetic acid which instantly inactivated the enzyme. Since alkylation is irreversible, the kinetics of the reaction can be followed by determination of the thioltransferase activity remaining after adding an excess of iodoacetic acid. Different concentrations of S-sulfocysteine were used to react with reduced pig liver thioltransferase at pH 6.8. (Fig. 22). In a period of 22 min, more than 95% of the thioltransferase activity was protected in the presence of 400 uM S-sulfocysteine, whereas only 40% of the activity was protected by 70 uM S-sulfocysteine. Since the reaction with substrate is probably reversible, and not a single step reaction, no attempt was made to explain the kinetics. However, the results clearly indicated that the protection of the enzyme activity was S-sulfocysteine concentration dependent (Fig. 22). The effect of pH on the reaction between the reduced enzyme and S-sulfocysteine was also investigated by carrying out the reaction at constant reactant concentrations at different pH. No apparent difference in the reaction as monitored by iodoacetic acid inactivation was observed at a pH range of 6.0 to 8.1 (Fig. 23). It is known that a

Fig. 22. Protection of thioltransferase activity by S-sulfocysteine from iodoacetic acid inactivation. Reduced pig liver thioltransferase (constant concentration of 70 uM) was incubated with S-sulfocysteine at room temperature in the presence of 0.1 M sodium phosphate buffer, pH 6.8. At various times, an aliquot of 10 ul was taken to mix with iodoacetic acid which give a final concentration of 5 mM. The inactivation by iodoacetic acid proceeded for 1 min. The thioltransferase activity was assayed after a 1000 fold dilution. The iodoacetic acid concentration in the assay mixture was 5 uM which did not affect the thioltransferase assay. S-sulfocysteine concentrations used are indicated as follow: ●, 70 uM; ○, 100 uM; ▲, 140 uM; ■, 400 uM.

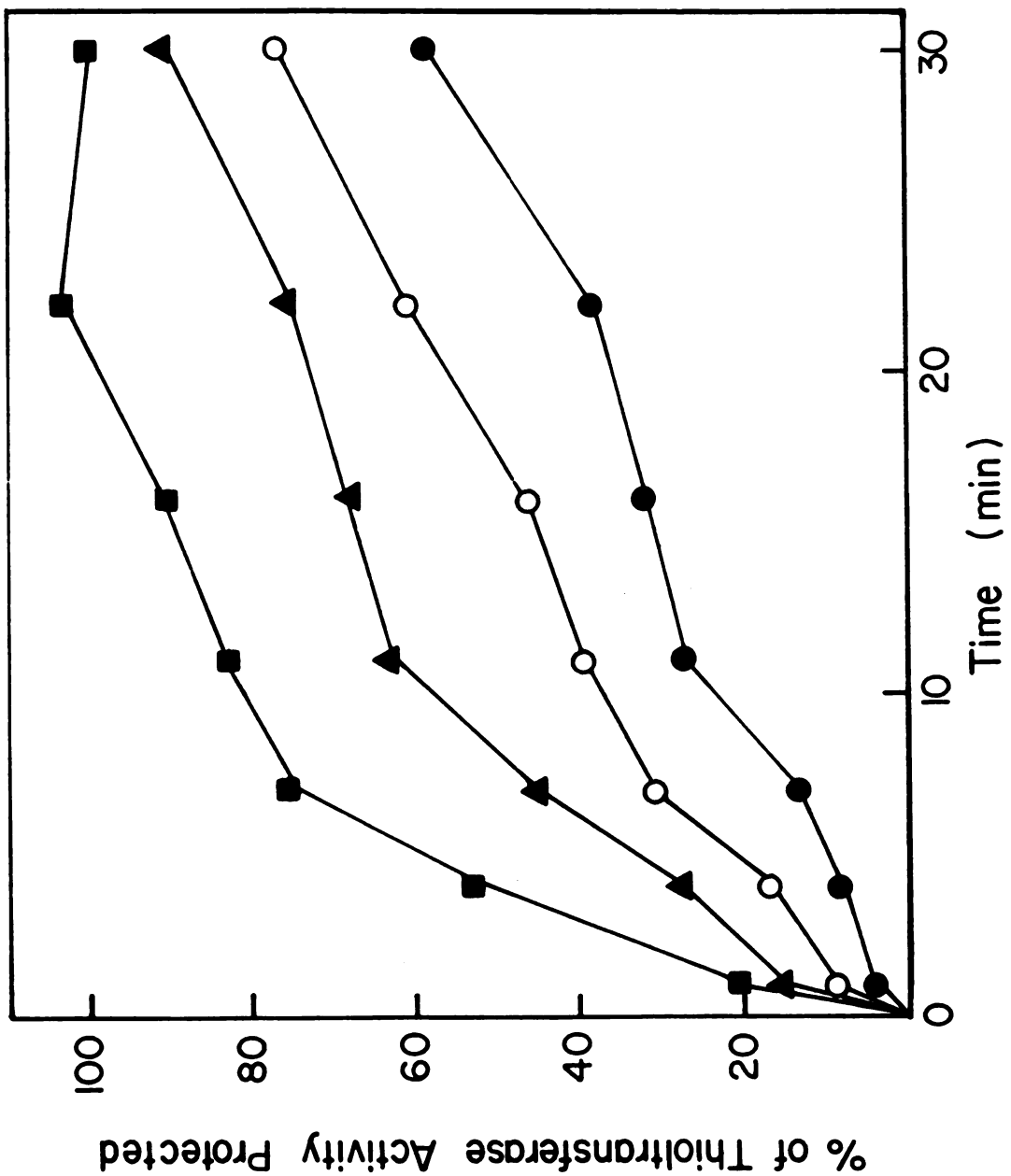
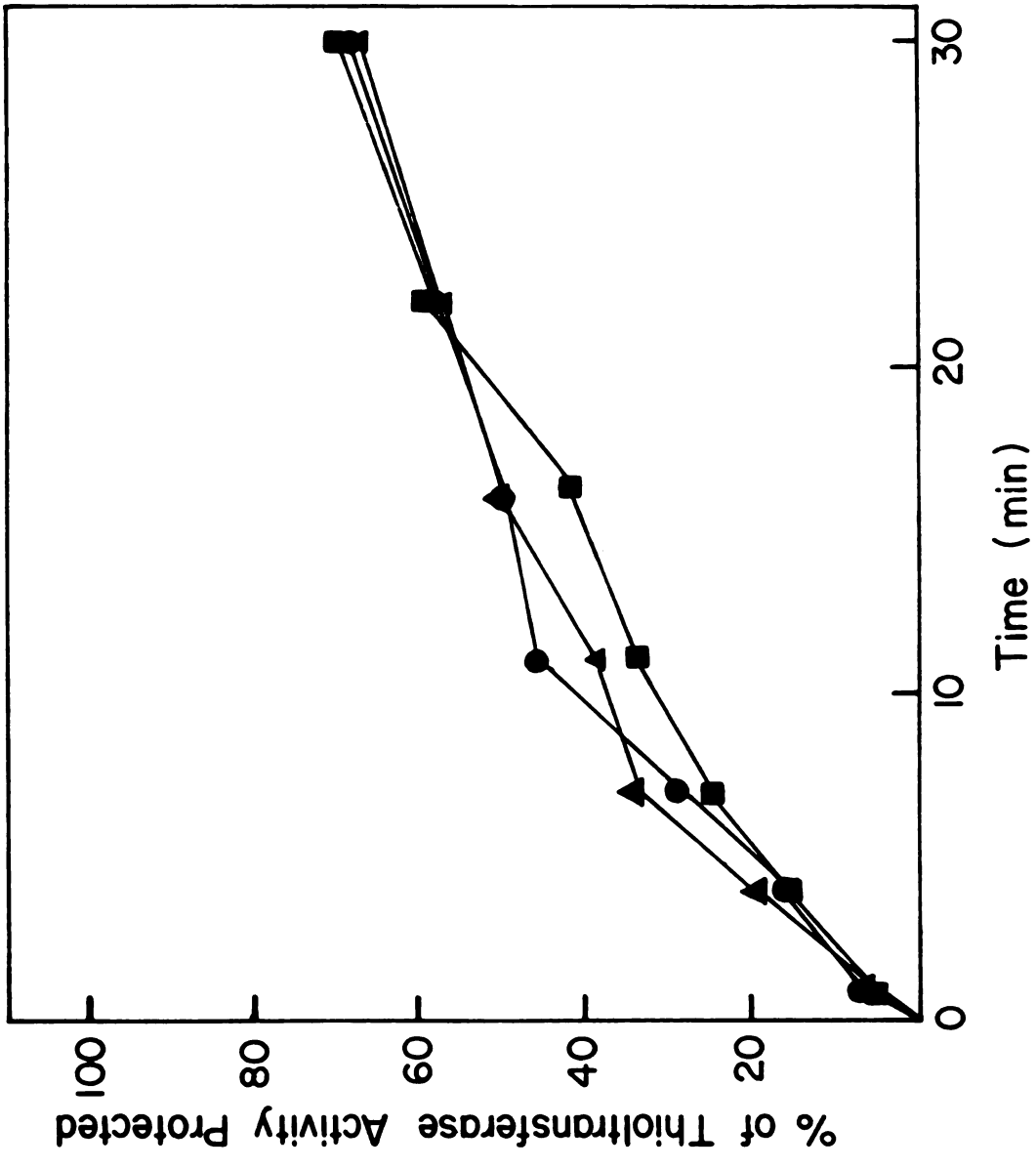

% of Thioltransferase Activity Protected
100
80
60
40
20
0
10
20
30
Time (min)

Fig. 23. Effect of pH on S-sulfocysteine protection of reduced pig liver thioltransferase activity from iodoacetic inactivation. Reduced enzyme (70 uM) was incubated with S-sulfocysteine (100 uM) in the presence of 0.1 M sodium phosphate buffer, at different pH values. At various times, an aliquot of 10 ul was taken to mix with iodoacetic acid which give a final concentration of 5 mM. The inactivation of the enzyme proceeded for 1 min. The dilution and enzyme assay are identical to those described in Fig. 22. The pHs used are indicated as follow: ●, pH 6.0; ▲, pH 7.5; ■, pH 8.1.


% of Thiolltransferase Activity Protected
100
80
60
40
20
0
10
20
30
Time (min)

thiolate anion is required to attack a disulfide bond in a typical reduction reaction (16). Since the active site sulfhydryl group of thioltransferase, Cys 22 has a pKa of 3.8 (Fig. 20), the active site sulfhydryl should be in its anion form throughout the physiological pH range used (6.0-8.1). It also follows that if the reduced enzyme reacts with its disulfide substrate by a nucleophilic attack, the reaction rate should be pH independent at pH 6 to 8.1. The results shown in Fig. 23 are consistent with such a reaction mechanism.

The same protocol was employed to follow the kinetics of the reaction between oxidized enzyme (intramolecular disulfide) and reduced glutathione. In this case, the enzyme could not be immediately inactivated by excess iodoacetic acid when the enzyme was treated with GSH. Thus, the oxidized enzyme was first incubated with GSH, either at a constant GSH concentration or a constant pH. To achieve an equilibrium state, the incubation proceeded for 30 min and then iodoacetic acid was added to the incubation mixture to give a concentration of 5 mM. The thioltransferase activity was assayed at different times. In the absence of GSH, the oxidized enzyme was not sensitive to iodoacetic acid (data not shown). The percentage of thioltransferase activity inactivated at different GSH concentration or different pH are given in Fig. 24 and 25. The inactivation of GSH treated enzyme by iodoacetic acid was both GSH concentration and pH

Fig. 24. Inactivation of GSH treated pig liver thioltransferase by iodoacetic acid. Different concentrations of GSH were used to react with oxidized enzyme (80 uM) for 30 min at room temperature in the presence of 0.1 M sodium phosphate buffer, pH 6.8. Iodoacetic acid was then added to the incubation mixture to give a concentration of 5 mM. At various times, thioltransferase activity was assayed after dilution. Iodoacetic acid concentration in the assay mixture was less than 5 uM which did not affect the thioltransferase assay. GSH concentrations used are indicated as follow: ●, 90 uM; ▲, 200 uM; ■, 1000 uM.

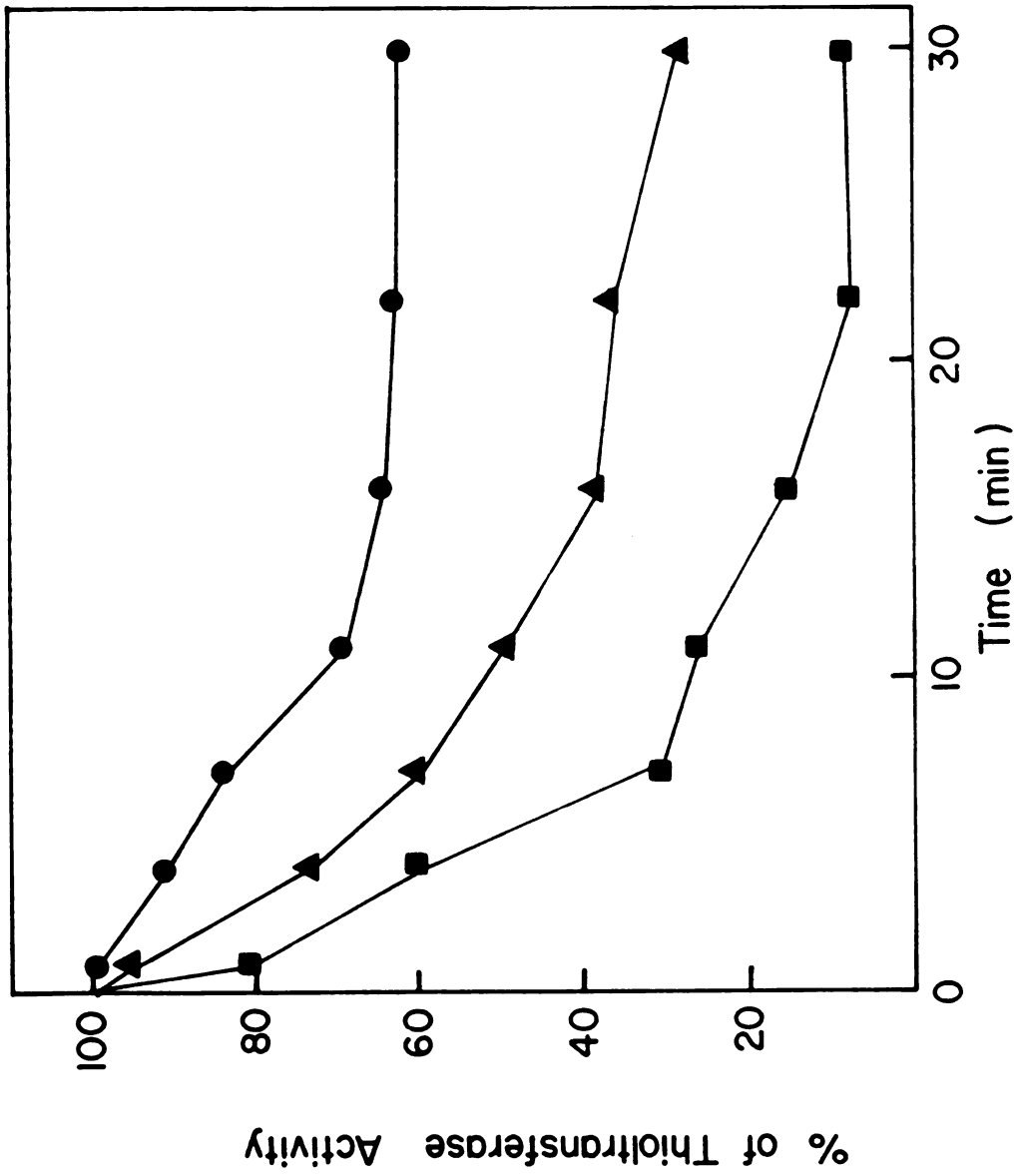
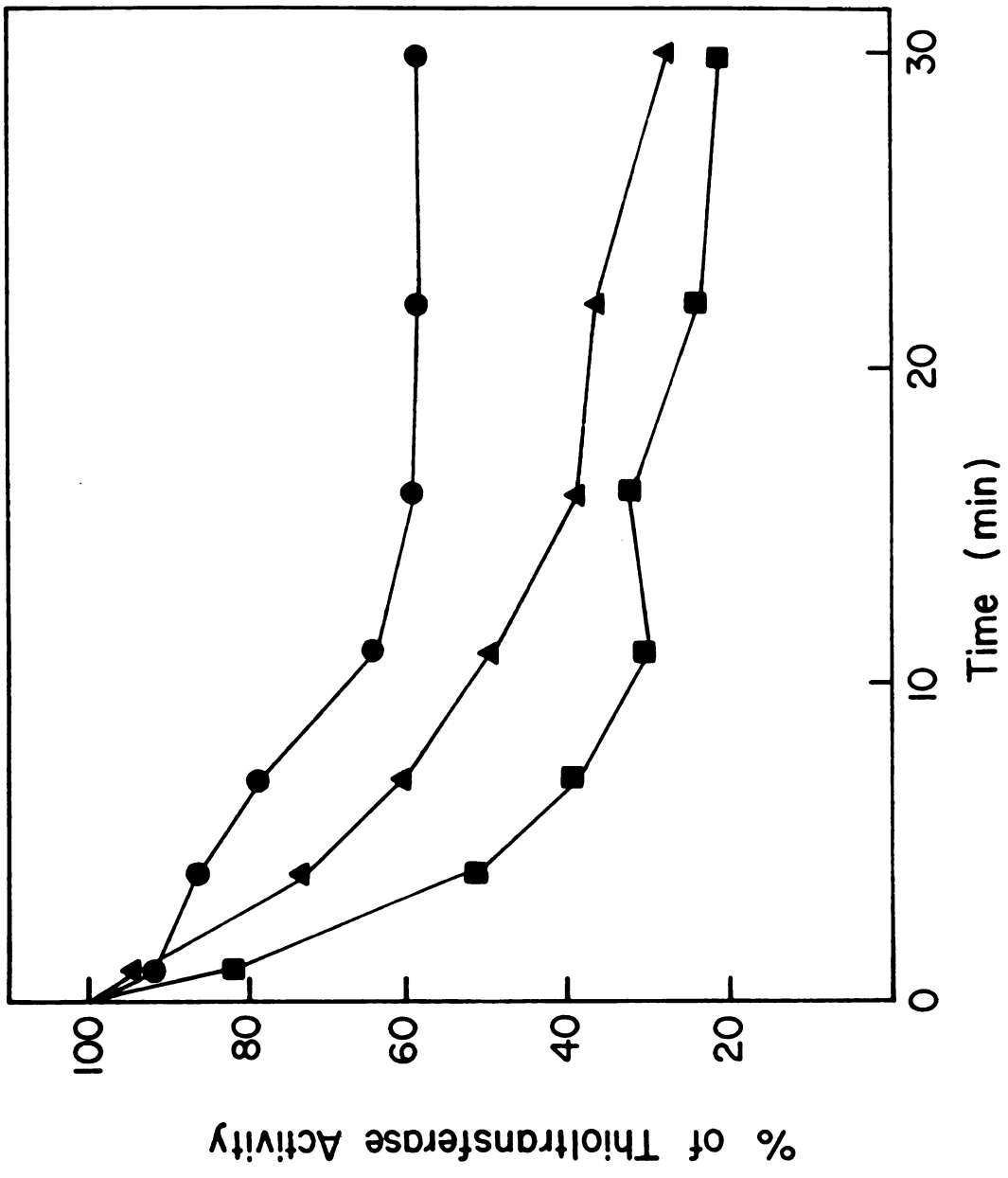
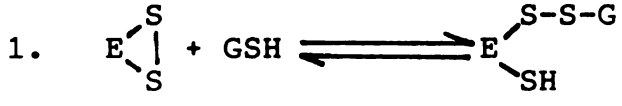
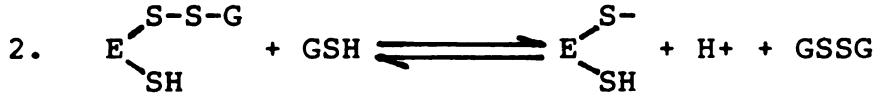

% of Thioltransferase Activity
100
80
60
40
20
0
10
20
30
Time (min)

Fig. 25. Effect of pH on iodoacetic acid inactivation of GSH treated pig liver thioltransferase. Oxidized enzyme (80 uM) was incubated with GSH (200 uM) for 30 min at room temperature in the presence of 0.1 M sodium phosphate buffer, at different pH. The enzyme was then alkylated and assayed under the same condition as described in Fig. 24. The pHs used are indicated as follow: ●, pH 6.0; ▲, pH 6.8; ■, pH 8.1.


% of Thioltransferase Activity
100
80
60
40
20
0
10
20
30
Time (min)

dependent. The interesting question is why the GSH treated enzyme could not be immediately inactivated by iodoacetic acid. One explanation is that because of noncovalent binding of GSH at the active site, the enzyme was protected from alkylation. If this was true, the inactivation rate of the enzyme by iodoacetic acid at high concentrations of GSH should be lower than that at low GSH concentrations. However, the results were completely opposite (Fig 24). Another possibility is the formation of a mixed disulfide between the active site cysteine and GSH after cleavage of the intramolecular disulfide of the enzyme by GSH as shown below, reaction 1. The mixed disulfide would then be reversibly reduced by GSH as shown in reaction 2.





Alkylation of the active site cysteine would force the equilibrium to shift to the right, and the equilibrium would be controlled by the GSH concentration. Thus, when the GSH concentration increases, the inactivation rate of the enzyme by iodoacetic acid would increase. At constant GSH concentration, the inactivation rate would also increase with increasing pH. This may be due to either a

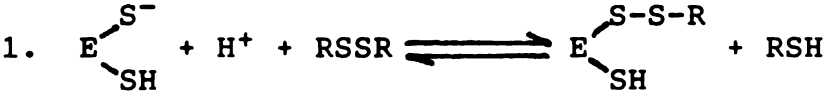
conformational change of the enzyme or to the dissociation of a proton from GSH or both. The complete mechanism of the reaction between oxidized thioltransferase and GSH will require further study.

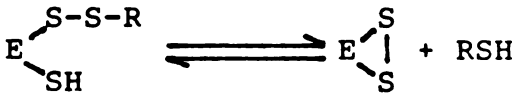
DISCUSSION

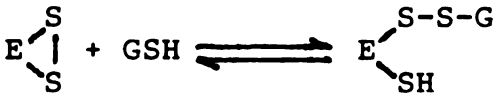
The alkylation of a thiol, as well as the reduction of a disulfide by thiols, requires a thiol anion which can initiate a nucleophilic attack (16). The ionization of a thiol is strongly influenced by neighboring group effects. The pKa values of cysteine thiols may be determined by their pH-dependent reactivity with an appropriate disulfide (16). The present work demonstrated that only Cys 22 of pig liver thioltransferase reacted with iodoacetic acid in a physiological pH range. From the studies reported here, the sulfhydryl group of Cysteine 22 has a pKa of approximately 3.8. It has been reported that the reaction rate of disulfide reduction by a thiol is electrostatically dependent (16). For example, positively charged disulfides react with thiol more rapidly than those with negative charge. Thus, compared with low molecular weight thiols, the ionization status of protein thiols are more strongly affected by their microenvironment. An unusual feature of the primary structure of pig liver thioltransferase is that there are no negatively charged amino acid residues from the N-terminus to residue 29, whereas, 5 basic amino acids were found. In particular, three positively charged amino acid residues, Lys 19, Arg 25, and Lys 26, surround Cys 22. While the primary structure of the protein may facilitate the low pKa of Cys

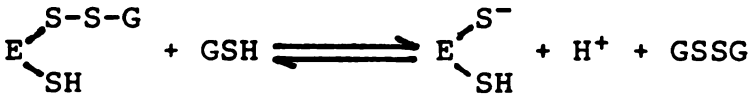
22, the three dimensional structure of the protein may also play an important role in the ionization of the sulfhydryl group of Cys 22.

Several proteins have been reported to catalyze sulfhydryl-disulfide interchange (18-21). The active site of thioredoxin has been well characterized with a sequence of -Trp-Cys^{32}-Gly-Pro-Cys^{35}-Lys-, which undergoes a thiol-disulfide interchange in the presence of the NADPH-dependent thioredoxin reductase system (11). The sulfhydryl group of cysteine 32 has a pKa of 6.7, which can effectively react with disulfides at a physiological pH. The location of the reactive thiol protrudes out from the surface of the protein making it accessible to other disulfide surfaces (22). The similar sequences, -Trp-Cys-Gly-His-Cys-Arg- in the protein disulfide isomerase (20) and -Thr-Cys-Pro-Tyr-Cys-Lys- in glutaredoxin (23), have been presumed to be the active site of these enzymes. However, the ionization properties of the thiols of these two enzymes have not been studied. I report here that the active site of thioltransferase has a sequence of -Thr-Cys^{22}-Pro-Phe-Cys^{25}-Arg-Lys-. A model for the action of the enzyme for a typical thiol-disulfide oxidoreduction is proposed as follow:



2. 

3. 

4. 

In the model above, RSSR and E represent disulfide substrate and enzyme, respectively. The sulfhydryl group of Cys 22 initiates a nucleophilic attack on the disulfide, resulting in an enzyme-substrate mixed disulfide complex. The mixed disulfide will be rapidly reduced by an intramolecular rearrangement in which deprotonated Cys 25 presumably in close juxtaposition cleaves the mixed disulfide bond creating the intramolecular enzyme disulfide bond. Then, the oxidized enzyme is reduced by GSH in a two step reaction. Though theoretically possible, it is unlikely that either Cys 78 or Cys 82 would take part in this intramolecular disulfide formation since they are likely to be a considerable distance from the active site. Furthermore, in E. coli thioredoxin or glutaredoxin, only two cysteines, analogous to Cys22 and Cys25, are required for catalysis. The next steps in the model are involved in the regeneration of the reduced enzyme by GSH for which two moles of GSH are required (Equations 3 and 4). Since the pKa of sulfhydryl group of Cys 22 is about 3.8, the initial nucleophilic attack on the disulfide by the

enzyme is probably very fast at a physiological pH. Reaction 2 is also assumed to proceed rapidly because of the rapid deprotonation of Cys 25 in the transient intermediate complex and to its close proximity to the initial mixed disulfide. Our previous work showed that pig liver thioltransferase had an optimum pH of 8.5 to 9, and thioltransferase activity was strongly dependent on [GSH] (9). In this chapter, it was found that the reaction between the reduced enzyme and a disulfide was not pH dependent whereas the reaction between oxidized enzyme and GSH was enhanced at higher pH (Fig. 23 and 25). These results support the belief that the rate limiting steps of the reaction catalyzed by thioltransferase are those reactions (equations 3 and 4) between oxidized enzyme and GSH. The detailed interaction between oxidized thioltransferase and GSH is still not clear. The fact that the oxidized enzyme, treated with 1 mM GSH for 30 min, could not be immediately inactivated by iodoacetic acid implies a complicated interaction between them that will require further study.

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CHAPTER V

SUMMARY AND FUTURE RESEARCH

During this Ph.D program, pig liver thioltransferase was purified to homogeneity by a novel procedure. This procedure has been successfully applied to the purification of calf liver and human placenta thioltransferase. The primary structure of pig liver thioltransferase revealed a high homology with calf thymus glutaredoxin. The active site amino acid of pig liver thioltransferase was identified as Cys 22, the sulfhydryl group of which has a pKa of 3.8. These results have opened several areas for further study of this enzyme.

Initially one may investigate the regulatory function of thioltransferase by modulating enzyme thiol-disulfide status. The unusual pKa of the enzyme endows thioltransferase with the potential to reduce protein disulfide by using GSH as a cosubstrate. By treating ^{35}S labeled cytosolic protein mixed disulfides with GSH alone or the thioltransferase system, my preliminary results indicated that radioactivity was removed only by the complete thioltransferase system. This result implied that some metabolic enzyme activity could be affected by thioltransferase system if their active site or allosteric sites contain a cysteine which can undergo thiol-disulfide oxidoreduction. It may be fruitful to investigate the interaction between thioltransferase and those enzymes sensitive to alkylation reagents.

The reason for the low pKa of the active site cysteine of the enzyme has been ascribed to the amino acid sequence

around the active site. To confirm this speculation, several amino acids near the active site could be changed by site-directed mutagenesis after cloning and expression of thioltransferase. Since the complete amino acid sequence of the enzyme is now known and antibodies against pig liver thioltransferase are available, this research should be ready to start.

In addition, preliminary results have shown that the concentration of thioltransferase is age dependent. To continue this work, a synchronized cell line could be used to monitor thioltransferase activity at different cell cycle stages. Furthermore, the regulation of the enzyme expression is a big open area to future study.

Another aspect of thioltransferase research is to clarified the relationship between thioltransferase and glutaredoxin. One of the last criteria remaining to justify the common identity of these two proteins is to see whether thioltransferase can be coupled to mammalian ribonucleotide reductase system. The purification of calf thymus ribonucleotide reductase for this purpose is now under investigation in this laboratory.