Identification and Characterization of the Functional Amino Acids at the Active Center of Pig Liver Thioltransferase by Site-directed Mutagenesis*

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By using site-directed mutagenesis techniques, the essential amino acids at the catalytic center of porcine thioltransferase (glutaredoxin) were determined. Seven oligonucleotides were designed, synthesized, and used to construct mutants, ETT-C22S, ETT-C25S, ETT-C25A, ETT-R26V, ETT-K27Q, ETT-R26V:K27Q, and ETT-C78S:C82S, by altering their codons in pig liver thioltransferase cDNA/M13mp18 clones. Each of the thioltransferases was purified to homogeneity and its dithiol-disulfide exchange, and dehydroascorbate reductase activities were compared with those of the wild-type (ETT). Evidence was obtained that CysZ2 was essential for catalytic activity, and the extremely low pKₐ value of its sulfhydryl group was facilitated primarily by ArgZ6. The role of LysZ7 at the active center was different from that of ArgZ6 and may be important in stabilizing the E-S intermediate by electrostatic forces. The second pair of cysteines, CysZ78 and CysZ92, nearer the C terminus, were not directly involved in the active center, but may play a role in defining the native protein structure. The replacement of the original Cys with a Ser at position 25 increased rather than decreased the enzyme activity, suggesting that the formation of an intramolecular disulfide bond between CysZ2 and CysZ92 is not necessary for the catalytic mechanism of the SerZ5 mutant, but does not rule out such a mechanism for the wild-type enzyme.

Thioltransferase was originally called glutathione-homocystine transhydrogenase by Racker (1) who discovered the enzyme in beef liver in 1955. In contrast, glutaredoxin was reported as a component in an alternate electron transport system for ribonucleotide reductase in mutant Escherichia coli lacking thioredoxin (2). As more thioltransferases and glutaredoxins were purified and characterized from different sources (1-11), the similarities between the two proteins became obvious. First, both enzymes could catalyze dithiol-disulfide exchange reactions in the presence of GSH (1,3,10,12). Second, amino acid sequence comparison of pig liver thioltransferase in E. coli in the unfused state with all activity in the unfused state with all activity and kinetic behavior analogous to the native enzyme (21). The heterologous expression system is efficient and suitable for making the soluble low molecular weight protein. In this paper, we describe the construction of seven mutant thioltransferases (glutaredoxins) by site-directed mutagenesis, their expression, purification to homogeneity, and relative thiol-disulfide exchange and dehydroascorbate reductase catalytic behavior.

EXPERIMENTAL PROCEDURES

RESULTS

Mutagenic Oligonucleotides and Site-directed Mutagenesis—Six of the seven oligonucleotides were designed for exchange of the amino acids located in the alleged active center. The seventh mutant replaced the 2nd pair of cysteines absent in procaryotic glutaredoxin, with serine residues. The oligonucleotides varied between 17-mer and 29-mer in length and are summarized in Table I. Each oligonucleotide was complementary to the nontranscribed thioltransferase cDNA strand in specific regions, except those designed mutant bases (underlined). The base changes of each mutant were confirmed by nucleotide sequencing in which each of the mutant cDNAs in M13mp18s was used as template (data not shown).

Expression and Purification of Mutant Thioltransferase—The seven mutant pig liver thioltransferase cDNAs of con-
firmed nucleotide sequence were subcloned into plasmid pKK232-2 between the unique Ncol and HindIII sites, separately. The seven newly constructed expression vectors, containing specific mutagenized thioltransferase cDNAs, were sequentially named pTT2 to pTT8 corresponding to the mutant protein products of ETT-C22S, ETT-C25S, ETT-C25A, ETT-R26V, ETT-K27Q, ETT-R26V-K27Q, and ETT-C78S-C82S. The expression vector for the wild-type enzyme (ETT) was named pTT1 previously (21). JM105 cells were transformed with the expression vectors pTT1 to pTT8 separately, and the wild-type and mutant thioltransferases were expressed during 6 h of isopropyl-1-thio-β-D-galactopyranoside induction.

Analysis of the crude extracts by immunoblotting demonstrated that the wild-type and mutant type pig liver thioltransferases were successfully expressed in E. coli with approximately equal efficiency (data not shown). All mutant enzymes were purified to homogeneity, as seen by a single band on SDS-PAGE (Fig. 1).

Thioltransferase Activity Comparison—The thiol-disulfide exchange activities of wild-type and mutant thioltransferases were compared based on the same amount of protein (0.4 µg) (Fig. 2). The activity of the wild-type thioltransferase (ETT) was defined as 100%, and, accordingly, the relative activities of the mutants were 0% for ETT-C22S, 110% for ETT-C25S, 32% for ETT-R26V, 67% for ETT-K27Q, 5% for ETT-R26V-K27Q, 9% for C25A, and 90% for ETT-C78S-C82S. As speculated, exchange of Cys with a Ser at position 22 of pig liver thioltransferase completely eliminated the enzyme activity. This is the first direct evidence revealing that Cys is the required active site amino acid residue of mammalian thioltransferase (glutaredoxin). It has been known that the sulfhydryl group of Cys has an extremely low pK, of 3.8 (20), and this property has been speculated to be facilitated by the two neighboring amino acids, Arg and Lys. Changing the Arg to a Val or the Lys to a Glu at the active center, we found a 68% or a 33% reduction in activity, respectively. But, if the two basic amino acids were changed simultaneously, only 5% of the wild-type enzyme activity remained. These results indicated that both basic amino acids strongly influenced the activity of the native enzyme. Interestingly, the replacement of Cys with a Ser at position 25 caused an increase rather than a decrease in enzyme activity, suggesting that the formation of an intramolecular disulfide bond between Cys and Cys is not the only possible mechanism for enzymatic catalysis. However, the substitution of the Cys with an Ala at this position caused a 91% reduction in activity, implying that an amino acid residue with a more polar side group, such as -CH2SH or -CH2OH, at this position is required for optimal enzyme activity. The second pair of cysteines, Cys and Cys, near the C terminus of the enzyme, can be substituted with serines without altering thiol-disulfide exchange activity more than 10%. The thioltransferase activity of the wild-type and the mutant enzymes were assayed over the pH range of 5.5–9.5 using different amounts of protein. The general pattern of thioltransferase activity dependence on pH for each enzyme was similar with low rates at acidic pH values and with maximum rates at pH 8.5–9.0 (Fig. 3). Thus, modifications at the active center did not change the optimum pH of the enzyme, excluding the total inactive mutant, ETT-C22S.

These results imply that the amino acid substitutions at the active center did not cause major conformational differences, and the activity changes were the result of the active site alterations. Future three-dimensional x-ray crystallographic analysis should establish the validity of this speculation.

The kinetic property of each thioltransferase with respect to the Cys-SO concentration is shown in Fig. 4. The v versus [S] plots of the wild-type and mutant thioltransferases showed non-Michaelis-Menten kinetics, i.e. at high substrate concentration, the enzyme activity was inhibited. The values of K values for these enzymes were estimated to be 0.5–0.8 mM. Our data clearly showed substrate (Cys-SO; or cystine) inhibition of the thioltransferases, and the products of thioltransferase action, cysteine and HSO, were inhibitors of glutathione reductase. But the latter inhibition could not be neglected in the current study because of the excess of glutathione reductase and the negligible concentration of cysteine and HSO at the initial stages of the reactions.

The DHA Reductase Activity—In the presence of GSH, thioltransferase can catalyze the reduction of DHA to ascorbic acid (28). The intrinsic DHA reductase activity of the wild-type and each of the mutant enzymes was measured as described under “Experimental Procedures” and compared with each other based on the same amount of protein (0.4 µg) (Fig. 5). With the activity of the wild-type enzyme defined as 100%, the relative activities of the mutants were 0% for ETT-C22S, ETT-R26V-K27Q, and ETT-C25A, 194% for ETT-C25S, 30% for ETT-R26V, 73% for ETT-K27Q, and 71% for ETT-C78S-C82S. Like the thiol-disulfide exchange activity, the ETT-C22S mutant had no detectable DHA reductase activity. This result indicates that Cys is very likely the catalytic site for both intrinsic activities. Compared with the thiol-disulfide exchange activity, ETT-C25S had a significantly greater DHA reductase activity, whereas there was no detectable activity for ETT-C25A. The evidence suggests that a serine is more favored than a cysteine at position 25, especially for DHA reductase activity. The DHA reductase activity of thioltransferase is less active in mutants ETT-R26V, ETT-K27Q, ETT-C78S, and ETT-R26V-K27Q than the corresponding thiol-sulfide exchange activity. The co-existence of the two basic amino acids, Arg and Lys, appear to facilitate both intrinsic activities of thioltransferase.

Isoelectric Focusing Analysis—The wild-type thioltransferase (ETT) and mutant thioltransferases were analyzed both in their reduced forms (Fig. 6A) and oxidized forms (Fig. 6B) by isoelectric focusing. The pl values of the reduced thioltransferases were 7.0 for ETT-C25S, ETT-C25A, and ETT-C78S-C82S, 7.5 for ETT-C25S, 5.8 for ETT-R26V, 6.1 for ETT-K27Q, and 5.3 for ETT-R26V-K27Q. In contrast, the pl values of the oxidized forms were approximately 8.0 for ETT, ETT-C25S, ETT-C25A, and ETT-C78S-C82S, and ETT-C78S-C82S, 7.5 for ETT-C25S, 5.8 for ETT-R26V, 6.1 for ETT-K27Q, and 5.3 for ETT-R26V-K27Q. There were two bands for oxidized forms of ETT-C25S and ETT-C25A (Panel B of Fig. 6). We speculate that the upper one is the unoxidized (reduced) form and the lower one is the oxidized form for both mutants.

The PK Values of Mutant and Wild-type Thioltransferases—An example of a plot of 1/(1/[T]) versus the time of reduction of 1.0 µM ETT-C25S and 60 µM iodoacetamide were incubated in 100 mM sodium phosphate buffer, pH 6.8, at room temperature. The apparent rate constant, kapp, was 5.5 mm−1 min−1 and the corresponding half-time, t1/2, was 3.0 min. Similar plots at various designated pH values for each enzyme were drawn (data not shown), and their kapp values were calculated. The PK values of the Cys

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1 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Cys-SO; S-sulfocysteine; DHA, dehydroascorbic acid; DTT, dithiothreitol; ETT, expressed thioltransferase; ETT-C22S, expressed mutant thioltransferase with an S substitution for the original C at position 22 (other mutants are represented similarly); HED, hydroxyethyl disulfide; HPLC, high performance liquid chromatography.

2 Y. Yang and W. W. Wells, unpublished data.
sulfhydril group for each enzyme was obtained from the midpoints of plots of $k_{\text{app}}$ versus pH (Fig. 8). For the expressed wild-type thioltransferase (ETT), the apparent rate constant was pH-dependent over the pH region of 3.0 to 4.5, whereas it was pH-independent between pH 4.5 to 8.5. The extremely low $k_{\text{app}}$ values below pH 3 indicated that Cys$^{27}$ was in the sulfhydril form (−SH) and not sensitive to the alkylation reagent. The increasing $k_{\text{app}}$ values between pH 3.0 and 4.5 signified that the deprotonation on the Cys$^{27}$ sulphydryl occurred in this pH range and that more thiolated forms became exposed to iodoacetamide. The unchanged $k_{\text{app}}$ values over the pH region of 4.5 to 5.5 implied that the maximum of alkylation reaction was reached and all Cys$^{27}$ side chains were in the thiolate form. The $pK_a$ value of Cys$^{27}$ of the wild-type recombinant thioltransferase was about 3.8, consistent with that of the native enzyme (20). Thus, the acetylation at the N terminus of the native pig liver enzyme has no influence on the $pK_a$ of Cys$^{27}$. The $pK_a$ values of the Cys$^{27}$ side chain were estimated to be 4.9 for ETT-C25S, 4.3 for ETT-K27Q, 5.9 for ETT-C25A, and 4.4 for ETT-C78S/C82S, all more basic than that of the wild-type. Substitution of the cysteine at position 25 with either a serine or an alanine caused a more basic shift of the $pK_a$ of Cys$^{27}$ than the changes at position 27 (ETT-K27Q) and position 78 and 82 (ETT-C78S/C82S). $pK_a$ as well as Arg$^{26}$ was originally speculated to facilitate the low $pK_a$ at Cys$^{27}$. However, in the mutant, ETT-K27Q, the $pK_a$ of Cys$^{27}$ was only slightly increased (approximately 0.5 pH unit) and the activity decreased by 33%, whereas a greater $pK_a$ increase occurred in the mutant ETT-C25S, and the enzyme activity was raised 10%. Thus, for ETT-K27Q, the 33% loss of enzyme activity was not the result of the slight $pK_a$ increase, but $pK_a$ likely played some other role in the enzyme catalytic mechanism. Currently, the precise function of Lys$^{27}$ is unknown, although one possibility is that this residue can stabilize the enzyme-substrate intermediate by ionic interactions between its positively charged side chain and a negatively charged group of the substrates, e.g., GSH. It is interesting that replacing Cys$^{27}$ with Ser$^{27}$ and with Ala$^{27}$, separately, resulted in different $pK_a$ changes at Cys$^{27}$ (1.1 and 2.1 pH units, respectively) and resulted in totally different activity alterations (10% increase versus 91% decrease, respectively). Compared with serine, the relatively more hydrophobic alanine replacing Cys at this position might disturb the local three-dimensional structure of the active center and cause Cys$^{27}$ to be less exposed. The exchanges of the two downstream cysteines, Cys$^{76}$ and Cys$^{86}$, with 2 serines had little influence on the $pK_a$ value of Cys$^{27}$. Accordingily, the low $pK_a$ of Cys$^{27}$ sulphydryl in the wild-type enzyme group is not facilitated by either Cys$^{25}$, Lys$^{27}$, or Cys$^{76}$ and Cys$^{86}$. In contrast, the amino acid responsible for the low $pK_a$ at Cys$^{27}$ was Arg$^{26}$. We could not measure the $pK_a$ value of Cys$^{27}$ with Cys$^{25}$, Val$^{26}$, and Arg$^{26}$ significantly decreased the deprotonation of the active site sulphydryl group. We conclude that the role of Arg$^{26}$ is to facilitate the low $pK_a$ of Cys$^{27}$, i.e., enhance its S-nucleophilicity, necessary for the thioltransferase catalytic reaction. Despite the apparent correlation between Cys$^{27}$SH $pK_a$ and enzyme activity, it is still presence of arginine at position 26, and enzyme activity, it is still possible that valine at position 26 may exert its effect on activity by blocking accessibility of Cys$^{27}$.

**DISCUSSION**

The primary structure of thioltransferase (glutaredoxin) has been reported for E. coli (31), calf thymus (15, 16), pig liver (13, 14), rabbit bone marrow (11), and, recently, yeast (32). All the proteins have an active site of Cys-Pro-Tyr (the pig enzyme)-Cys-, while the three mammalian enzymes contain an additional pair of cysteines near the C terminus (Fig. 9). The sequences of the two regions containing the cysteine pairs are highly conserved in thioltransferases (glutaredoxins). The first region is the active center for each enzyme, and in the sequences in this region are identical except that a Phe instead of a Tyr was found in the pig enzyme, and only one basic amino acid is located in this region for the E. coli and yeast enzymes. In the second conserved region near the C terminus, the sequences are the same in the three mammalian enzymes except that a Thr is replaced by a Ser in the rabbit enzyme. Despite the lack of the extra pair of cysteines, the E. coli and the yeast enzyme still have considerably high sequence identity with the mammalian proteins in this region suggesting that the second conserved region might have a structural function. However, our data showed that the replacement of the second pair of cysteines in the pig enzyme affected its activity only slightly. It is interesting that similar cysteine pair distributions occur in thioredoxin, another low molecular weight protein catalyzing various thiol-disulfide exchange reactions, i.e., there are two pairs of cysteines in mammalian enzymes (33) and only one pair in the active site of the bacterial and yeast thioredoxins (34, 35).

The present work provides the first direct evidence for the identification of the essential amino acids in the active center of a mammalian thioltransferase (glutaredoxin). Since the substitution of certain original amino acids in the mutants caused large pH shifts, the pH of the buffers used in the purifications had to be modified (Table II). The $pK_a$ value of the sulfhydryl group of Cys$^{27}$ ($pK_a = 3.8$) is much lower than that of normal cysteine ($pK_a = 8.5 \pm 0.5$) (31), and Cys$^{27}$ was speculated to be the active site of thioltransferase (19). This was directly confirmed by results of changing Cys$^{27}$ to Ser$^{27}$, which totally eliminated the enzyme activity. These data also showed that the amino acids Arg$^{26}$ and Lys$^{27}$ are required for optimal enzyme activity since exchange at any of these positions generally decreased the activity with the exception that replacement of Cys$^{25}$ with Ser$^{26}$ increased rather than decreased the enzyme activity. This discovery necessitated a re-evaluation of the enzyme mechanism for the mutant enzymes not capable of forming an intramolecular disulfide. Individual replacement of Arg$^{26}$ or Lys$^{27}$ with Val$^{26}$ or Gln$^{27}$ led to a relative 32% or 67% enzyme activity, respectively, but altering the 2 basic residues together with two neutral amino acids caused a cooperative loss in activity.

We did not change the two amino acids, Pro and Phe, between the 2 cysteines at the active center. However, two such studies in T4 (36) and E. coli thioredoxin (37) were reported recently. Joelson et al. (36) constructed three mutants, CGPC, CVPC, and CGYC, at the active site of T4 thioredoxin which has the sequence, CVYC, and found no significant changes in the enzyme activity. Gleason et al. (37) constructed two mutants, CGGRC and CAC, at the native protein active site, CGPC, of E. coli thioredoxin by altering the size and demonstrated that the longer mutant lost 86% of its activity, whereas the one with a shorter chain had no activity. The mutation studies implied that the distance rather than the specific amino acids substituted between the two active site cysteines is the more important factor. The double loop at the active site of the oxidized enzyme seems to have been the preferred choice during evolution. However, as we
demonstrate here, a serine at position 25 might have been expected to enjoy an evolutionary advantage.

The present results indicate that exchange of some amino acids, especially the charged residues (e.g., Cys, Arg, and Lys) at the active sites caused the pI shifts of the proteins. Normally, when a protein is in its native folded form, the pI value of the protein is the sum of the net charges on the surface of the molecule (38). In the present case, all thioltransferases, analyzed on isoelectric focusing gel, were in their native folded forms. Thus, the pI values of these proteins should reflect the total surface charges of the native molecules, and the pI shift should follow the changes of the surface charged groups. The substitution of Cys with a Ser at position 22 caused a pI change in both reduced forms (0.5 pH unit) and oxidized (0.4 pH unit) forms, whereas, the same substitutions at positions 25, 78, and 82 had no influence on the pI values (Fig. 6). These results provide additional evidence that Cys$\beta$ is the only cysteine residue in its thiolated form (-S-) at a physiological pH and that it is exposed or partially exposed to the molecular surface, whereas Cys$\alpha$, Cys$\gamma$, and Cys$\delta$ are either in their sulfhydryl forms (-SH) or buried in the protein core. The three-dimensional structures of E. coli (39) and T4 (40) thioredoxins have shown that their active sites are parts of a protrusion of the molecule. So, it is possible that the active site of thioltransferase (glutaredoxin) is on the surface of the molecule, and only this location of the active center can explain how the low molecular weight enzyme interacts with its protein substrates, such as pyruvate kinase (41) and ribonucleotide reductase (42). Certainly, this proposal needs further support from the three-dimensional structure of thioltransferase, and such studies are now in progress.

Striking pI shifts were observed in both reduced forms and oxidized forms when the 2 basic amino acid residues were replaced by neutral amino acids either individually or together. Compared with the wild-type enzyme (ETT), mutants ETT-R26V, ETT-K27Q, and ETT-R26V/K27Q have more acidic pI values and the double mutant has the most acidic pI. These pI changes apparently resulted from the loss of the positively charged Arg or Lys or both in the active center possibly located on the molecular surface. We showed that thiol-disulfide exchange and DHA reductase activity of the mutants ETT-R26V, ETT-K27Q, and ETT-R26V/K27Q are significantly decreased, and, thus, the two basic amino acids, Arg$\beta$ and Lys$\beta$, must be involved in each catalytic activity.

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Table 1

| Sequence of oligonucleotide primer (5' to 3') | Location in PLT cDNA (nt) | Amino acid substitution |
|--------------------------------------------|--------------------------|------------------------|
| 1. **AGCTGGTGGGTTGGAACGC**              | 82 to 102               | C25S                   |
| 2. **GCGGGGAAGTCGTCTITC**                | 91 to 107               | C25A                   |
| 3. **CCGCCAAGECTCITTTCTC**               | 91 to 108               |                        |
| 4. **GGAACACGULTTTCTCTC**                | 94 to 111               | R26V                   |
| 5. **AGACGTCTPTCTGTCTC**                 | 97 to 107               |                        |
| 6. **GCAAGACCULTPTCTCTTC**               | 94 to 113               | R26Y:K27Q              |
| 7. **ACATTCTCTCATGATCCCTGCTACTA**        | 249 to 268              |                        |

b) The loci refers to the original sequences in PLT cDNA complementary to the relative oligonucleotide primers.

Table 2

| Oligo No. | Vector Name | Protein Product | Buffer A pH | Buffer B pH |
|-----------|-------------|-----------------|-------------|-------------|
| 1          | pET1        | ETT             | 6.5         | 7.5         |
| 2          | pET2        | ETT:CC5S        | 6.5         | 7.5         |
| 3          | pET3        | ETT:CC25S       | 6.2         | 7.1         |
| 4          | pET4        | ETT:CC2A        | 6.2         | 7.2         |
| 5          | pET5        | ETT:CC254       | 6.0         | 7.0         |
| 6          | pET6        | ETT:K27Q        | 6.0         | 7.0         |
| 7          | pET7        | ETT:R26VK2Q     | 5.0         | 6.0         |
| 8          | pET8        | ETT:CC78S:CC2S  | 6.5         | 7.5         |

Fig. 1 SDS-PAGE analysis of the purified pig liver thioltransferase. Each of the mutant thioltransferase was purified as described in Experimental Procedures. The purified samples (1 μg) were analyzed on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Lanes 1 to 9 were Bio-Rad low-molecular weight protein standards, ETT, ETT:CC25S, ETT:CC2A, ETT:CC254, ETT:K27Q, ETT:R26VK2Q, ETT:CC78S:CC2S, respectively.
Active Site of Pig Liver Thioltransferase

Fig. 2. Thiol-disulfide exchange activity comparison of the mutant and wild-type pig liver thioltransferases. Using the same amount of protein (0.4 μg), the thiol-disulfide activity of each purified enzyme was measured by the standard assay system which contained 0.5 mM GSH, 1.4 units of glutathione reductase, 2.5 mM β-mercaptoethanol, 0.05 mM NADPH, and 137 mM sodium phosphate buffer, pH 7.5. The relative activity of mutant thioltransferases as based on the wild-type enzyme activity that was defined as 100%.

Fig. 3. Optimum pH of mutant Thioltransferases. TT activity dependence on pH was compared among the mutants and wild-type proteins. The activities of wild-type and mutant thioltransferases were measured by the standard assay system (Experimental Procedures). In each respective assay, 0.25 μg ETT, 0.20 μg ETT-C255, 1.0 μg ETT-C25A, 0.90 μg ETT-R26V, 0.4 μg ETT-K270, 3.0 μg ETT-R26V-K270, and 0.27 μg ETT-C78S-C82S were used. Each value is the average of three separate experiments.

Fig. 4. Kinetic behavior of mutant Thioltransferases. Thioltransferase activity dependence on S-sulfocysteine concentration was measured for the wild-type and mutant enzymes. The standard assay (Experimental Procedures) was used with increasing S-sulfocysteine concentrations. In each separate assay, 0.35 μg ETT, 0.35 μg ETT-C255, 3.0 μg ETT-C25A, 0.85 μg ETT-R26V, 0.65 μg ETT-K270, 4.5 μg ETT-R26V-K270, and 0.36 μg ETT-C78S-C82S were used. Each value is the average of three different experiments.

Fig. 5. Comparison of the DNA reductase activity of thioltransferases. The DNA reductase activities of the wild-type and mutant thioltransferases were measured based on the same amount of protein (0.4 μg) as described in Experimental Procedures. The activity of the wild-type enzyme was defined as 100%, and the relative activities of mutant enzymes were compared.

Fig. 6. Isoelectric focusing of mutant thioltransferases. The purified wild-type and mutant thioltransferases (1 μg) were treated with 10 mM DTT (Panel A) or 10 mM H2O2 (Panel B) for 30 min at room temperature, and the pI values were measured on a Servalyte PAG gel, pH 3-10, according to the manufacturer’s instructions. The gel was stained with Coomassie Brilliant Blue. The reduced forms in Panel A, lanes 1 to 9, were served as marker proteins, ETT, ETT-C255, ETT-C25A, ETT-R26V, ETT-K270, ETT-R26V-K270, ETT-C25A, and ETT-C78S-C82S, respectively. The oxidized forms in Panel B, lanes 1 to 10, were pI standards, ETT, ETT-C255, ETT-C25A, ETT-R26V, ETT-K270, ETT-R26V-K270, ETT-C25A (<0.1 μg), and ETT-C78S-C82S and ETT-C25A, respectively.
Active Site of Pig Liver Thioltransferase

Fig. 7. A plot of the alkalization reaction between the same concentrations (10 μM) of reduced ETT-Cys5 and Cys255. This reaction was performed at room temperature in 100 mM sodium phosphate buffer, pH 6.8. The values of reduced thioltransferase at various times were determined by enzyme activity assay after dilution (see text for details). The second order apparent rate constant, kapp, of the reaction was 9.5 nM⁻¹min⁻¹ and the half-time was 3.0 min.

Fig. 8. pH dependence of second order apparent rate constant of the reactions between each of the reduced thioltransferases (10 μM) and e.indomethacin (50 μM). The log values of the reactions at various pHs were obtained for each enzyme as described in Fig. 7 and Experimental Procedures.

Fig. 9. Comparison of thioltransferase amino acid sequences in two conserved regions. The amino acid sequences of thioltransferases from E. coli, yeast, calf thymus, rabbit bone marrow, and pig liver are compared in two cysteine containing regions. Alignment is based on the active site and the locations of the two regions are indicated with the number for each enzyme. The sequences other than the two regions are omitted. Identical amino acids are outlined by solid lines, whereas dashed lines denote differences.