Catalytic Mechanism of Thioltransferase*

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To evaluate potential catalytic mechanism for thioltransferase thiol-disulfide exchange reactions, seven pig liver mutants were constructed by site-directed mutagenesis. All the expressed enzymes, including wild-type and mutants with the exception of the inactive mutant, ETT-C22S, were variably inhibited by iodoacetamide, and similar results were obtained when these enzymes were preincubated with GSH. However, when preincubated with S-sulfocysteine or hydroxyethyl disulfide, the activity of the enzymes was totally or partially protected against inhibition by iodoacetamide, with the exception of the mutants, ETT-C22S and ETT-C25A. When simultaneously pretreated with GSH and S-sulfocysteine, all enzymes were highly protected. Isoelectric focusing analysis of the above preincubation mixtures showed that different enzyme-substrate intermediates occurred. Using radioactively labeled substrates, [U-14C]cystine and [glycine-2-3H] GSH, enzyme-substrate intermediates were detected. These data indicate that reduced thioltransferase reacts first with disulfide substrates, then with a thiol substrate, e.g. GSH. The formation of either enzyme-substrate mixed disulfide or protein intramolecular disulfide protected the enzyme from inactivation by iodoacetamide. Based on the experimental results, alternative methods of the catalytic mechanism for thioltransferases are proposed.

Thioltransferase, also called glutaredoxin, has been known for more than 35 years (1). This low molecular weight, heat-stable cytosolic enzyme is widely distributed in bacteria, yeast, and animals, and its thiol-disulfide exchange catalytic properties and primary structure have been studied extensively (2-7). Coupled with glutathione reductase and NADPH, thioltransferase catalyzes reversible thiol-disulfide exchange reactions in the presence of GSH. The whole reaction system may play an important role in the regulation of enzyme activity and/or in maintaining a normal cellular thiol/disulfide ratio (8-11). The alkylation reagents, iodoacetic acid (IAA) and iodoacetamide (IAM), irreversibly inactivate thioltransferase by reaction with a single sulfhydryl group at its active site, Cys2 (12), in a pH-dependent manner (7). Since alkylation of a thiol occurs only in the deprotonated thiolate form (12, 13), the formation of a mixed disulfide or an intramolecular disulfide at the active site of thioltransferase should make the enzyme insensitive to alkylating reagents.

In an accompanying article (21), we described the construction of seven mutant pig liver thioltransferases by site-directed mutagenesis, directly identified the essential amino acids at the active center, and characterized these mutants. Exchange of the cysteine with a serine at position 25 caused an increase rather than a decrease in both thiol-disulfide exchange activity and dehydroascorbic acid (DHA) reductase activity of the enzyme and prompted a more complete study of the catalytic mechanism of native and mutant thioltransferases.

In this article, we report the results of studies to investigate the catalytic reactions of thioltransferase by (i) alkylation inhibition, (ii) isoelectric focusing of enzyme-substrate complexes, and by (iii) reactions with radioactive labeled substrates.

EXPERIMENTAL PROCEDURES

Materials
L-Cystine, dithiothreitol (DTT), GSH, iodoacetamide, and iodoacetic acid were from Sigma; 2-hydroxyethyl disulfide (HED) was from Aldrich; L-[U-14C]cystine (300 mCi/mmol) and [glycine-2-3H] GSH (1 Ci/mmol) were purchased from Du Pont-New England Nuclear; isoelectric focusing gel and PI marker proteins were from Serva; Safety-solve and 2,5-diphenyloxazole (PPO) were from Research Products International Corp.; dimethyl sulfoxide was obtained from J. T. Baker, Inc.

Preincubation and Inhibition Studies of Thioltransferases
The reduced wild-type and mutant pig liver thioltransferases were prepared as described previously (see the accompanying paper (21)). Each of the reduced enzymes (0.06 mM) was incubated with 0.12 mM IAM in the presence of 100 mM sodium phosphate buffer, pH 7.5, at room temperature. At various times, samples were withdrawn, and the thiol-disulfide exchange activity of each enzyme was assayed using the standard system (5). The IAM inhibition experiments were also performed after preincubating each enzyme (0.06 mM) with GSH (0.5 mM), Cys-SO3- (2.5 mM), Cys-SO3- (2.5 mM) + GSH (0.5 mM), and HED (2.5 mM), separately, in the presence of 100 mM sodium phosphate buffer, pH 7.5, for 15 min at room temperature.

Isoelectric Focusing Analysis
The wild-type thioltransferase (ETT) and mutants ETT-C22S and ETT-C25S, 3 μg each, were treated with 2.5 mM HED, 2.5 mM cystine, 2.5 mM Cys-SO3-, and 0.5 mM GSH + 2.5 mM Cys-SO3-, separately, in 100 mM sodium phosphate buffer, pH 7.5, for 15 min at room temperature. Each of the incubation mixtures (in a total volume of 10 μl) was then analyzed directly by isoelectric focusing gel electrophoresis as described previously (14).

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

14C]Cystine—Each of the reduced wild-type and mutant thiol-
The inhibition of such enzyme activity by IAM is dependent upon the availability of the free reduced enzyme with the thiolate form of CysZ2. In order to test the possible catalytic mechanism, each of the wild-type and mutant thioltransferases, only the sulhydryl group of CysZ2 is the target of the alkylating reagents at pH 7.5 (7, also see the accompanying paper (21)). When incubated with IAM, more than 85% of the thiol-disulfide exchange activities of the wild-type enzyme (ETT) and the mutants, ETT-C25S and ETT-C25A, ETT-K27Q, and ETT-C78S:C82S were inhibited, whereas the mutants of ETT-R26V and ETT-R26V:K27Q still had approximately 70% and 90% activity remaining, respectively (Fig. 1). The latter two mutants, each with a Val exchanged for an Arg at position 26, were not sensitive to IAM inhibition at pH 7.5 since they had lost the ability to facilitate the deprotonation of their Cys2' side chain. The inactivation of other expressed enzymes by IAM was a function of the extent of the available thiolate side chain (–CH2S–) of Cys2'. These results were consistent with those of the previous pK, measurement experiments described in an accompanying paper (21).

A typical thioltransferase-catalyzed thiol-disulfide exchange reaction involves the enzyme, a disulfide substrate (cystine, HED, or a thiosulfate ester, e.g. Cys-SO3-), and GSH. The inhibition of such enzyme activity by IAM is dependent upon the availability of the free reduced enzyme with the thiolate form of Cys2'. In order to test the possible catalytic mechanism, each of the wild-type and mutant thioltransferases, except ETT-C25S, was pretreated with either GSH, Cys-SO3-, or Cys-SO3- + GSH, separately, and then incubated with IAM (Fig. 2). Preincubation of these enzymes with GSH led to results similar to those when reduced enzymes were incubated directly with IAM (Fig. 2, left). These results implied that GSH alone had little effect on reduced enzyme inactivation except for slight promotion of the alkylating reaction, presumably by reducing previously air-oxidized

forms of the enzymes. However, when pretreated with the disulfide-like substrate, Cys-SO3-, all enzymes were totally or partially protected from inactivation by IAM except the two mutants, ETT-C25S and ETT-C25A which lack cysteine 25 (Fig. 2, middle). Identical results were obtained using L-cystine instead of Cys-SO3- (data not shown). The mutants, ETT-R26V, ETT-R26V:K27Q, and ETT-C78S:C82S retained their total activity by pretreatment with Cys-SO3- or L-cystine, possibly by the formation of an intramolecular disulfide between cysteines 22 and 25, which prevented IAM reaction with Cys2'. The wild-type and mutant, ETT-K27Q were partially protected by the disulfide substrate, suggesting that there were still some free reduced enzyme molecules available to IAM. The mutants, ETT-C25S and ETT-C25A, lacking the ability to establish an intramolecular disulfide bond within their active center, were inhibited by IAM even after the initial formation of a mixed disulfide bond between

Thioltransferase Mechanism

PREINCUBATION WITH INHIBITION STUDIES—For pig liver thioltransferase, only the sulphydryl group of Cys2' is the target of the alkylating reagents at pH 7.5 (7, also see the accompanying paper (21)). When incubated with IAM, more than 85% of the thiol-disulfide exchange activities of the wild-type enzyme (ETT) and the mutants, ETT-C25S, ETT-C25A, ETT-K27Q, and ETT-C78S:C82S were inhibited, whereas the mutants of ETT-R26V and ETT-R26V:K27Q still had approximately 70% and 90% activity remaining, respectively (Fig. 1). The latter two mutants, each with a Val exchanged for an Arg at position 26, were not sensitive to IAM inhibition at pH 7.5 since they had lost the ability to facilitate the deprotonation of their Cys2' side chain. The inactivation of other expressed enzymes by IAM was a function of the extent of the available thiolate side chain (–CH2S–) of Cys2'. These results were consistent with those of the previous pK, measurement experiments described in an accompanying paper (21).

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the enzyme and the substrate. However, when simultaneously preincubated with Cys-SO$_3^-$ and GSH, all thioltransferases were relatively well protected, including the two mutants lacking Cys$^{25}$ (Fig. 2, right). We believe that the co-existence of saturation levels of both substrates promoted the formation of the relatively stable enzyme-SG mixed disulfide at Cys$^{22}$ (see discussion below).

The enzymes were also pretreated with HED, and, except for the two Cys$^{25}$-substituted mutants, all others were fully protected against inactivation by IAM (Fig. 3). Because of the two exceptions, this protection appears to be acquired by the formation of intramolecular disulfides. Similar studies were done using the native pig liver thioltransferase, and this enzyme was fully protected when preincubated with cystine, Cys-SO$_3^-$, or HED, but not with GSH (data not shown) in agreement with Gan and Wells (16). The only difference between the native and the recombinant enzyme is that the former has an N-acetylated N terminus (14). The different sensitivity to IAM of the two enzymes when preincubated with cystine or Cys-SO$_3^-$ suggests that one possible role of acetylation at the N terminus is protection of the native enzyme against unwanted physiological thiol alkylation reactions.

**Isoelectric Focusing Analysis**—The inhibition studies described above could not reveal what kinds of enzyme-substrate intermediates were formed. We have demonstrated in an accompanying paper (21) that the exchange of Cys$^{22}$ with a serine caused a significant protein pI shift of 0.5 pH unit. The wild-type enzyme ETT, mutant ETT-C25S, and mutant ETT-C78S:C82S were differentially treated with DTT, HED, cystine, Cys-SO$_3^-$, and IAM as described under “Experimental Procedures” and analyzed by an isoelectric focusing gel (Fig. 4). Lanes 2 to 8 were the wild-type enzyme, ETT, treated with DTT, HED, cystine, Cys-SO$_3^-$, and IAM as described above. The PI value for each of the enzymes, 3 μg (either in the free state or in the modified state), was measured on a Servalyt Precoat isoelectric focusing gel, according to the manufacturer’s instructions. The sample in each lane is indicated.

IAM-treated mutant ETT-C78S:C82S, respectively. Normally, when treated with 10 mM DTT or HED, the enzyme is fully reduced (thiolate) or oxidized (intramolecular disulfide), respectively, and the pI values are widely different from each other (Fig. 4, lanes 2 versus lane 3, lane 9 versus lane 10). However, when treated with cystine or Cys-SO$_3^-$, the pI values of ETT and ETT-C25S lay between those of their reduced and oxidized forms, i.e. the enzymes were neither in their thiolate nor intramolecular disulfide forms. Instead, they appeared to be in mixed disulfide forms, i.e. the charged side chain (–CH$_3$S$^-$) of cysteine 22 had been modified by the chemical nature of the mixed disulfide. In addition, more then one band existed in Cys-SO$_3^-$-treated samples (lane 5 and lane 12), suggesting that both substrate components, i.e. cysteine and bisulfite, can form a mixed disulfide or thiosulfate ester with ETT and ETT-C25S. The top, middle, and bottom bands represent reduced free enzyme, enzyme-SO$_3^-$, and enzyme-cysteine disulfide forms, respectively. The mixed disulfide formed between enzyme and GSH was also observed in ETT, ETT-C25S, and ETT-C78S:C82S in the presence of disulfide substrates (the upper bands of lanes 6, 7, and 12, and lane 13), and their pI values were fortuitously the same as those of the reduced enzymes due to the net negatively charged peptide, GSH. The pI values of IAM-treated enzymes were quite similar to those of oxidized ones. This gave additional evidence that IAM reacted with Cys$^{22}$ eliminating its negative charge as in the case of intramolecular disulfide formation. For mutant ETT-C78S:C82S, the pI value was equal to that of the oxidized form when treated with either cystine or Cys-SO$_3^-$.

**Radioactive Labeling Studies**—For further testing of the mixed disulfides between the enzyme and its substrates, two radioactively labeled substrates, [14C]cystine and [3H]GSH, were used to track the reaction progress. The details of the labeling experiments were described under “Experimental Procedures,” and the results are listed in Table I. Enzyme-substrate intermediates were detected, since radioactivity was measured in the collected G-25 protein fractions both before and after the concentration of the enzymes. The mutant, ETT-C25S, had the highest specific radioactivity (counts/min/μg) both in [14C]cystine-labeled samples or in [3H]GSH-labeled samples. No labeling by [3H]GSH was detected in mutant ETT-C22S due to the absence of the active site Cys$^{25}$. When labeled by [14C]cystine, there was no radioactivity...
Wild-type and mutant pig liver thioltransferases were incubated with \([^{14}C]\)cysteine, \([\text{glycine-2-}^{3}H]\)GSH, or Cys-SO; + \([^{14}C]\)GSH in the presence of 100 mM sodium phosphate buffer, pH 7.5, for 20 min at room temperature, and the excess radioactive-labeled substrates were removed by Sephadex G-25, and the samples were concentrated under conditions described in the text. The radioactivity of each enzyme was counted by liquid scintillation spectrometry, and the specific radioactivity was calculated.

### Table I

**Radioactive labeling of thioltransferases**

<table>
<thead>
<tr>
<th>Thioltransferases (0.3 mM)</th>
<th>Substrates</th>
<th>cpm/μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type ETT (0.6 mM)</td>
<td>L-[(^{14}C)]cysteine</td>
<td>114</td>
</tr>
<tr>
<td>ETT-C25S</td>
<td>([^{14}C])GSH</td>
<td>114</td>
</tr>
<tr>
<td>ETT-C25S + IAM (^{+})</td>
<td>Cys-SO; + ([^{14}C])GSH</td>
<td>114</td>
</tr>
<tr>
<td>ETT-R26V</td>
<td>ND</td>
<td>114</td>
</tr>
<tr>
<td>ETT-K27Q</td>
<td>ND</td>
<td>114</td>
</tr>
<tr>
<td>ETT-C78S:C82S</td>
<td>ND</td>
<td>83</td>
</tr>
</tbody>
</table>

\(^{+}\) ND, not determined.

ETT-C25S was pretreated with IAM, and then with L-[\(^{14}C\)]cysteine.

![MW (K)](Image)

**FIG. 5. Autoradiography of ETT and ETT-C25S.** Wild-type (ETT) and mutant (ETT-C25S) thioltransferases were treated with radioactively labeled substrates, \([^{14}C]\)cysteine or \([^{14}C]\)GSH, as described under "Experimental Procedures." Lanes 1 to 3 were ETT-treated with \([^{14}C]\)cysteine, Cys-SO; + \([^{14}C]\)GSH, and \([^{14}C]\)GSH, respectively. Lanes 4, 5, and 7 were ETT-C25S treated with \([^{14}C]\)cysteine, Cys-SO; + \([^{14}C]\)GSH, and \([^{14}C]\)GSH, respectively, whereas lane 6 was ETT-C25S pretreated with IAM followed by \([^{14}C]\)cysteine.

**DISCUSSION**

A thiol-disulfide exchange reaction is actually a nucleophilic ionic displacement that takes place either spontaneously or enzymatically, in vitro or in vivo (17, 18). The key aspect of this reaction is the existence of a thiolate anion as an attacking nucleophile (11). The extremely low pK, at the active site, cysteine 22, facilitates the catalytic action of thioltransferase. Alkylating reagents (e.g. IAA and IAM) inhibit thioltransferase-catalyzed exchange reactions, by binding to the sulphydryl group of cysteine 22 (7), but this kind of inhibition only happens when cysteine 22 is in its thiolate form. Whenever cysteine 22 is in either the -SH, mixed disulfide, or intramolecular disulfide forms, the enzyme is no longer sensitive to the alkylating reagent. Thus, substrate protection of the enzyme from the inhibition by an alkylating reagent, as well as radioactive labeling of the enzyme, makes it possible to test the nature of enzyme-substrate intermediates and further to provide evidence for the nature of the catalytic mechanism of thioltransferase.

Based on the data presented in this study, a model of the catalytic mechanism for thioltransferase is proposed as follows in Scheme 1. In scheme 1, E, S\(^{+}\), SH, and OH represent thioltransferase, the thiolate anion of cysteine 22, the sulphydryl group of cysteine 25, and the hydroxyl group of Ser\(^{79}\) of mutant ETT-C25S, respectively, whereas RSSR, RSH, GSH, and GSSG represent disulfide substrate, reduced product, glutathione, and glutathione disulfide, respectively.

In this model, the thiolate anion of the cysteine 22 of the reduced enzyme attacks the disulfide bond of RSSR to form an enzyme-mixed disulfide and a substrate thiol derivative (reaction 1). At this point, the enzyme may form an intramolecular disulfide bond between cysteines 22 and 25 releasing the reduced half-substrate thiol, and the enzyme intramolecular disulfide bond is displaced by GSH to form an enzyme-glutathione-mixed disulfide (reactions 2 and 3). Alternatively, GSH directly displaces the half-substrate to form the enzyme-glutathione-mixed disulfide (reaction 4). In either case, a second GSH molecule attacks the mixed disulfide to yield GSSG and regenerate the reduced enzyme (reaction 5). This model is consistent with the experimental results described herein. First, the order of the thioltransferase-catalyzed reaction is support by the data, in which the enzyme was not protected by GSH against IAM inhibition (Fig. 2, left) and could not be labeled by \([^{14}C]\)GSH (Table I and Fig. 5), in the absence of a disulfide substrate. These results suggest that the reduced enzyme is not protected from alkylation by GSH. However, GSH did bind to and protect the enzyme against IAM inhibition in the presence of disulfide substrate (Fig. 2, right, Table I, and Fig. 5) implying that the reduced enzyme reacts with a disulfide substrate first, followed by GSH. The differential protection of the wild type and mutant enzymes by disulfide substrate against IAM inhibition (Fig. 2, middle) can be explained with this model. In the absence of GSH, the reaction progresses only through reactions 1 and 2. The full protection against IAM inhibition in mutants, ETT-R26V, ETT-R26V-K27Q, and ETT-C78S:C82S is likely due to the formation of an intramolecular disulfide bond, which is more stable than the enzyme-SR-mixed disulfide bond. The complete inhibition of the two mutants without cysteine 25 (ETT-C25S and ETT-C25A) by IAM can be explained on the basis that reaction 1 is reversible, and IAM could remove the enzyme from the reaction by reacting with reduced free enzyme and forming a dead end reaction.
complex, i.e. pull the reaction to the left (reaction 6). For ETT and ETT-K27Q, the mixed disulfide formed in reaction 1 was competitively replaced either by the -SH group of Cys\(^{55}\) to form an intramolecular disulfide (reaction 2) or by the products to reverse the direction of reaction 1. In this situation, the rate of the reversal of reaction 1 was much slower, i.e. less free enzyme was available to IAM (reaction 6), and these two enzymes were only partially inactivated by IAM. Simultaneous preincubation of the enzymes with Cys-SO\(_2\)\(^{-}\) and GSH yielded much stronger protection of the enzymes, including the mutants ETT-C25S and ETT-C25A (Fig. 2, right). In addition, the enzymes could be labeled by [\(^{35}\)S]GSH in the presence of Cys-SO\(_2\)\(^{-}\). These results indicated the formation of enzyme-SG-mixed disulfide and provide credence to reactions 3 and 5 or 4 and 5 of the proposed model. This newly formed enzyme-SG-mixed disulfide bond may be relatively more stable than the enzyme-half-substrate disulfide bond (i.e. enzyme-SR). It is likely that during the preincubation, the enzyme-catalyzed reaction had reached a steady state in which the enzyme was virtually saturated by substrates, forming predominantly mixed and intramolecular disulfides, so that when IAM was added, little free reduced enzyme was available. However, IAM gradually pulled some of the enzyme molecules from the system at a very slow rate (reaction 6) since partial inhibition of the activity was observed. The existence of the enzyme-substrate intermediates has been confirmed by isoelectric focusing analysis of the thioltransferases pretreated with substrates and by radioactive labeling of the enzymes.

For a native protein, a pl value is normally the sum of its total molecular surface charges, and, therefore, the modification of any surface charge would change the pl value of the protein (19). In a companion paper (21), we have shown the pl differences between the reduced (treated with DTT) and oxidized (treated with HED) thioltransferases. In the current study, when treated with cystine, the wild-type enzyme (ETT) had a pl different from either the reduced (thiolate) form or oxidized (intramolecular disulfide) form (Fig. 4, lane 4) and there were two such pl forms for the Cys-SO\(_2\)\(^{-}\)-treated enzyme (Fig. 4, lane 5). In addition, the enzyme was labeled by [\(^{35}\)S]cysteine (Table I and Fig. 5), which gave evidence for the presence of an enzyme-SR intermediate. The existence of an enzyme-SG complex was also provided in the same way. For mutant, ETT-C78S:C82S, an intramolecular disulfide is formed when treated with disulfide substrates, since it has the same pl as that of the oxidized form (HED-treated), is not sensitive to IAM and is not labeled by [\(^{35}\)S]cysteine. Thus, we believe that, during the catalytic reactions, the formation of an intramolecular disulfide is an optional reaction dependent on the substrate involved. In the absence of GSH, whether reaction 2 proceeds or not depends on the strength of the -CH\(_2\)S\(^{-}\) nucleophilicity of the sulhydryl group of Cys\(^{55}\) and the thiol of the first product, RSH or RS\(^{-}\) (16, 18). If the former is stronger (e.g. in the case of HED as substrate), an intramolecular disulfide is formed (reaction 2). If the latter is stronger (i.e. cysteine or H-SO\(_2\)\(^{-}\) derived from Cys-SO\(_2\)\(^{-}\) as substrate), the reaction will be equilibrated as in reaction 1. In the presence of GSH, the reaction proceeds to reaction 5 from reaction 1 via either reactions 2 and 3 or reaction 4 to complete a catalytic cycle. When treated simultaneously with Cys-SO\(_2\)\(^{-}\) and GSH, there were two pl-mixed disulfide forms (i.e. enzyme-cysteine and enzyme-SG) for ETT (Fig. 4, lane 7), whereas there was only one pl form (i.e. enzyme-SG) for ETT-C25S (Fig. 4, lane 13). Therefore, the efficiency for displacement of the half-cystine from the first mixed disulfide by GSH is greater for ETT-C25S than for ETT. This might explain why this mutant enzyme has increased thiol-disulfide exchange and DHA reductase activities. Thus, in this model, reaction 3 or 4 may be the rate-limiting reaction.

The mechanism of the DHA reductase activity of thioltransferase is not established. However, Cys\(^{55}\) is likely to be the active site for both intrinsic enzymatic activities, and the mechanism of DHA reductase activity is presumably similar to that of a thiol-disulfide exchange reaction, i.e. a thiohemiketal intermediate, instead of a mixed disulfide, followed by displacement with GSH as shown in Scheme 2 (20). Studies are underway to further explore the hypotheses.

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REFERENCES
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