Identification and Reactivity of the Catalytic Site of Pig Liver Thioltransferase*

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The active site cysteine of pig liver thioltransferase was identified as Cys22. The kinetics of the reaction between Cys22 of the reduced enzyme and iodoacetic acid as a function of pH revealed that the active site sulphydryl group had a pKₐ of 2.5. Incubation of reduced enzyme with [1-1⁴C]cysteine 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]cysteine, suggesting an intramolecular disulfide formation.

The results suggested a reaction mechanism for thioltransferase. The thiolated Cys22 first initiates a nucleophilic attack on a disulfide substrate, resulting in the formation of an unstable mixed disulfide between Cys22 and the substrate. Subsequently, the sulphydryl group at Cys22 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.

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 alkylating 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 Escherichia coli, thioredoxin, has 2 cysteine residues located near the N terminus at positions -Cys47-Gly-Pro-Cys35- which participate in the active site of the enzyme (10). Furthermore, kinetic studies of the reactivity of the sulphydryl groups toward iodoacetic acid and iodoacetamide as a function of pH revealed that the pKₐ of Cys22 was 6.7, whereas that of Cys22 was 9.0 (11). In a companion paper (12), we have shown that pig liver thioltransferase contains 2 cysteine pairs with sequences of -Cys22-Pro-The-Cys25- and -Cys18-He-Gly-Gly-Cys26-, respectively. However, it has not yet been established which of these dithiol pairs are involved in the catalytic events.

In this article, we report that the active center of pig liver thioltransferase is located at cysteine 22. The sulphydryl group of this cysteine has a pKₐ of 2.5. The reduced enzyme was oxidized by [1-1⁴C]cysteine to form an intramolecular disulfide enzyme which was resistant to iodoacetic acid inactivation. These observations may suggest a possible mechanism for thioltransferase catalysis.

EXPERIMENTAL PROCEDURES

Materials—Iodo[1⁴C]acetic acid with a specific activity of 6.25 mCi/mmol was from ICN Radiobiocemicals. Ni-[1⁴C]cysteine with a specific activity of 74 mCi/mmol was from Research Products International Corp. Reduced glutathione, dithiothreitol, L-cysteine, iodoacetic acid, and glutathione reductase were purchased from Sigma. Sephadex G-25 and a Mono Q HR 5/5 HPLC column were from Pharmacia. A reversed-phase C18 HPLC column (Micro Pak, 0.4 × 30 cm) was from Varian. S-Sulfocysteine was prepared by the method of Segel and Johnson (13), and the product was recrystallized three times from aqueous ethanol. All the other reagents are either HPLC or analytical grade.

Thioltransferase Activity Assay—The enzyme was assayed as described previously (8). Briefly, the reaction mixture (500 µl) contained 0.5 mM GSH, 1.2 units of glutathione reductase, 2.5 mM S-sulfocysteine, 0.35 mM NADPH, 0.137 mM sodium phosphate buffer, pH 7.5, and the enzyme to be assayed. The reaction proceeded at 30 °C, and thioltransferase activity was measured spectrophotometrically at 340 nm.

Preparation of Reduced Pig Liver Thioltransferase—The homogenous enzyme was purified by a method described previously (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 × 45 cm) which was equilibrated and eluted with water. The protein was collected through a pipette 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/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 [1⁴C]cysteine at an enzyme concentration of 0.42 mM and a cysteine 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 cysteine on a Sephadex G-25 column (1 × 45 cm) which was equilibrated and eluted with water. The protein was lyophilized.

Carboxymethylation—Protein was carboxymethylated with iodoacetate as described by Gracy (14). Excess iodoacetic acid was removed by Sephadex G-25 chromatography as described above.

Cryptic 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 a companion paper (12).

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‡The abbreviation used is: HPLC, high performance liquid chromatography.
Sequencing of the Active Center Peptide—The methods applied in the sequencing of the peptide were described in a companion paper (12).

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 iodo[1-14C]acetic 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 companion paper (12). The results of radioactivity counting of the tryptic peptides showed that only peptide T3 was labeled by iodo[1-14C]acetic 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 I indicated that incorporation of 14C was sharply increased at residue 9 which corresponds to cysteine 22 of the protein. The total counts/min of the residues recovered before residue 9 were 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 iodo[1-14C]acetic acid-labeled enzyme was digested by chymotrypsin, and the peptides were separated as described (12). It was found that peptide C3, containing only 1 cysteine, Cys72, and terminating at Phe24 (12), was the only peptide labeled by [1-14C]iodoacetic acid.

Kinetics of the Reaction between Cys22 and Iodoacetic Acid—Since alkylation of Cys22 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 Cys22 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:

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

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 \([TT-0] - [TT-CM]\) 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. 1). 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. 2. The apparent rate constants between pH 5 and 6.6 were pH-dependent, whereas they increased between pH 5 and 3, and decreased below pH 3. The rate constant was near 0 at pH 6.5.

**Table I**

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>Amino acid</th>
<th>Yield</th>
<th>cpm × 10^4</th>
<th>cpm × 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total</td>
<td>per nmol</td>
</tr>
<tr>
<td>R1</td>
<td>Val</td>
<td>9.3</td>
<td>2.3</td>
<td>0.2</td>
</tr>
<tr>
<td>R2</td>
<td>Val</td>
<td>9.2</td>
<td>2.3</td>
<td>0.2</td>
</tr>
<tr>
<td>R3</td>
<td>Val</td>
<td>8.7</td>
<td>2.2</td>
<td>0.3</td>
</tr>
<tr>
<td>R4</td>
<td>Phe</td>
<td>11</td>
<td>2.1</td>
<td>0.2</td>
</tr>
<tr>
<td>R5</td>
<td>Ile</td>
<td>6.4</td>
<td>2.5</td>
<td>0.4</td>
</tr>
<tr>
<td>R6</td>
<td>Lys</td>
<td>4.9</td>
<td>2.2</td>
<td>0.3</td>
</tr>
<tr>
<td>R7</td>
<td>Pro</td>
<td>2.1</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>R8</td>
<td>Thr*</td>
<td></td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>R9</td>
<td>Cys</td>
<td>1.0</td>
<td>401</td>
<td>410</td>
</tr>
<tr>
<td>R10</td>
<td>Pro</td>
<td>0.8</td>
<td>189</td>
<td>236</td>
</tr>
<tr>
<td>R11</td>
<td>Phe</td>
<td>0.7</td>
<td>78</td>
<td>111</td>
</tr>
<tr>
<td>R12</td>
<td>Cys</td>
<td>0.4</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>R13</td>
<td>Arg</td>
<td>0.2</td>
<td>8.5</td>
<td>42</td>
</tr>
</tbody>
</table>

* The yield was not determined.

**Fig. 1.** 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] represents the reduced enzyme concentration calculated from the remaining thioltransferase activity.

**Fig. 2.** 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. 1. Blank thioltransferase activity was not affected when the pH decreased over the indicated range.
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 $pK_a$ 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 (11) that the apparent rate constant of iodoacetic acid with no negative charge was 20-fold higher than that of iodoacetic acid at pH 7.2, in similar studies with thioredoxin. 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 in agreement with an estimated $pK_a$ of 2.5 for the Cys$^{22}$ sulfhydryl group (see below). The interpretation of the above kinetics 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.

The protein labeled at different pH was subjected to chymotryptic digestion and the chymotryptic peptides were 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. These studies indicate that the enzyme Cys$^{22}$ has a $pK_a$ of approximately 2.5.

**Disulfide Protection of the Enzyme from Iodoacetate 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:

1. $E + \text{RSSR} \rightarrow E$ + RSSR
2. $E + 2 \text{H}^+ + \text{RSSR} \rightarrow E + \text{RSSR}$
3. $E + \text{RSSR} \rightarrow E + \text{RSSR}$

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 separate 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. 3), ruling out mechanism 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 sodium dodecyl sulfate-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 mechanism described in reaction 3, namely the formation of an intramolecular disulfide bond.

**Table II**

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

<table>
<thead>
<tr>
<th>pH</th>
<th>Moles carboxymethyl groups incorporated per mol of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>0.04</td>
</tr>
<tr>
<td>2.0</td>
<td>0.10</td>
</tr>
<tr>
<td>3.0</td>
<td>0.49</td>
</tr>
<tr>
<td>4.0</td>
<td>0.37</td>
</tr>
<tr>
<td>5.0</td>
<td>0.21</td>
</tr>
<tr>
<td>6.0</td>
<td>0.22</td>
</tr>
</tbody>
</table>

**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 $pK_a$ 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 $pK_a$ of approximately 2.5. 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
oxidoreduction is proposed as follows:

\[
E, \text{ site of thioltransferase has a sequence of } -\text{Thr-CysZ2-Pro-Phe-} \]

enzymes have not been studied. We report here that the active site of these enzymes has been presumed to be the active site of these enzymes. However, the ionization properties of the thiol residues of these two enzymes have not been studied. We report here that the active site of thioltransferase has a sequence of -Thr-CysZ2-Pro-Phe-CysZ5-Arg-Lys-. The low pKₐ of the sulfhydryl group of CysZ2 has been characterized with a sequence of -Trp-CysZ3'--Lys-, which undergoes a thiol-disulfide interchange (18-21). The active site of thioredoxin 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-Pro-Cys3'-Lys-, which undergoes a thiol-disulfide interchange, are anticipated to play an important role in the ionization of the cysteine group of CysZ2.

Several proteins have been reported to catalyze sulfhydryl-disulfide interchange (18-21). The active site of thioredoxin in 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, 3 positively charged amino acid residues, LysZ2, ArgZ2, and Lys29, surround CysZ2. While the primary structure of the protein may facilitate the low pKₐ of CysZ2, the three-dimensional structure of the protein may also play an important role in the ionization of the sulfhydryl group of CysZ2.

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-CysZ2-Pro-Tyr-Cys-Lys-, and enzymes have not been studied. We report here that the active site of these enzymes has been presumed to be the active site of these enzymes. However, the ionization properties of the thiol residues of these two enzymes have not been studied. We report here that the active site of thioltransferase has a sequence of -Thr-CysZ2-Pro-Phe-CysZ5-Arg-Lys-. The low pKₐ of the sulfhydryl group of CysZ2 suggests that thioltransferase will have a relatively negative Em, although these measurements have not yet been made. A model for the action of the enzyme for a typical thiol-disulfide oxireduction is proposed as follows:

\[
E + H^+ + RSSR \rightleftharpoons E + RSH \quad (1)
\]

\[
E + S-S-R \rightleftharpoons E + SH \quad (2)
\]

\[
E + GSH \rightleftharpoons E + SH + GSSG \quad (3)
\]

\[
E + GSH \rightleftharpoons E + H^+ + GSSG \quad (4)
\]

In the model above, RSSR and E represent disulfide substrate and enzyme, respectively. The sulfhydryl group of CysZ2 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 CysZ2, presumably in close juxtaposition, cleaves the mixed disulfide bond, creating the intramolecular enzyme disulfide bond. Although theoretically possible, it is unlikely that either CysZ2 or CysZ4 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 2 cysteines, analogous to CysZ2 and CysZ4, are required for catalysis. The next steps in the model are involved in the regeneration of the reduced enzyme by GSH for which 2 mol of GSH are required (Equations 3 and 4). Since the pKₐ of the sulfhydryl group of CysZ2 is about 2.5, 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 CysZ4 in the transient intermediate complex and by 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). Whether the proposed Reactions 3 and 4 are rate-limiting for the catalytic mechanism of thioltransferase remains to be determined.

Acknowledgments—We wish to thank Dr. Young Moo Lee and Melanie Markel for valuable assistance in active site peptide sequencing.

REFERENCES


![FIG. 3. Interaction between [1-14C]cystine and pig liver thioltransferase. The solid circles and triangles represent thioltransferase activity and counts/min of the fractions, respectively. For details, see “Experimental Procedures.”](image-url)