Three forms of a thiol proteinase inhibitor were isolated from rat liver cytosol. The monomeric inhibitor (pI 5.2) (TPI-1) formed a complex with cathepsin H even in the absence of reducing agents. The inhibitor with pI 5.0 (TPI-2) was inactive in the absence of reducing agents but was converted to an active inhibitor on addition of reducing agents such as dithiothreitol, GSH, cysteine, or 2-mercaptoethanol. The dimeric inhibitor (TPI-D) with an intermolecular disulfide bridge was also inactive and was converted to the active monomeric inhibitor on addition of dithiothreitol. TPI-2 is most likely a mixed disulfide with glutathione. One (Cys-3) of two cysteine residues exposed on the surface of the molecule of TPI-2 is involved in the formation of a mixed disulfide, and the other cysteine residue (Cys-64) is buried in the molecule. The activity of rat liver thiol proteinase inhibitor may possibly be regulated by formation of a protein mixed disulfide or by reduction of the mixed disulfide.

Endogenous inhibitors of thiol proteinases have been found in a variety of mammalian tissues (1–3) and in sera (2, 4–6). Intracellular inhibitors of lysosomal thiol proteinases, such as cathepsin B, H, and L, were recently identified and partially purified from rat lung and hog kidney by Lenney et al. (7). We have purified a thiol proteinase inhibitor from rat liver by papain affinity chromatography and have reported the characteristics of the purified inhibitor, the wide distribution of the inhibitor in various organs, its localization in the cytosol fraction, and its difference from inhibitors in serum (8–10). Recently, the complete sequence of the inhibitor from rat liver was determined (11); it consists of 98 amino acid residues with cysteinyl residues at positions 3 and 64.

During purification of the inhibitor, we found three fractions with inhibitory activity. Each of them was then purified by Sephadex G-75 and DEAE-cellulose chromatography. On polyacrylamide gel electrophoresis these three forms of the inhibitor showed different mobilities in the absence of reducing agents such as dithiothreitol but the same mobility in the presence of a reducing agent and on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. In the present study, we examined the relation of these three forms to the oxidation-reduction state of their sulfhydryl groups and to the inhibitor activity. Results showed that the monomeric inhibitor with a pI of 5.0 (TPI-2) is a mixed disulfide, possibly with glutathione. TPI-2 and the dimeric inhibitor with an intermolecular disulfide bridge were inactive and did not form a complex with cathepsin H in the absence of thiol compounds. The third form, TPI-1 was active even in the absence of thiol compounds.

This paper reports on the characteristics of the three forms of the inhibitor and discusses the possibility that changes in the oxidation-reduction state regulate activity of thiol proteinase inhibitor in intact cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Wistar rats weighing 200–300 g were used. CNBr-activated Sepharose 4B and Sephadex G-75 were from Pharmacia. DEAE-cellulose (DE-52) was from Whatman. Sodium iodoacetate, iodoacetamide, dithiothreitol, 5,5'-dithio-bis-(2-nitrobenzoate), and BrCN were obtained from Nakarai Chemicals. Homocysteine, cystamine dihydrochloride, and guanidine hydrochloride were from Wako Pure Chemical Industries. Reduced [glycine-2-3H]glutathione (5 Ci/mmole) and [14C]ICl, COOH (11 mCi/mmol) were obtained from New England Nuclear.

**Enzyme and Enzyme Assays**—Cathepsin H was prepared from rat liver by a modification of the method of Kirschke et al. (12). Papain (Type III) was obtained from Sigma. Cathepsin H and papain were assayed with benzoyl-arginine-2-naphthylamide as substrate by the method of Barrett (13). The reaction mixture in 1.0 ml, containing 5 μmol of substrate, added as a solution in 50 μl of dimethyl sulfoxide, 100 μmol of potassium phosphate buffer, pH 6.0, 2 μmol of EDTA, 4 μmol of cysteine, and an appropriate amount of enzyme, was incubated at 37°C for 10 min.

**Inhibitor Assay**—The inhibitor was assayed as described previously (9).

**Preparation of a Carboxymethylated Papain Affinity Column**—Papain (200 mg) was activated with 4 mM cysteine and 1 mM EDTA in 60 ml of 0.1 M potassium phosphate buffer, pH 6.0, for 20 min at room temperature and then treated with 200 mM iodoacetate. The solution was dialyzed against three changes of 0.1 M NaHCO3, and shaken for 2 h at room temperature with 30 ml of preswollen CNBr-activated Sepharose 4B. The gel was then treated with 0.1 M glycine-NaOH buffer, pH 8.0, for 2 h at room temperature and washed successively with 0.5 M NaCl in 0.1 M NaHCO3, pH 8.3, 0.5 M NaCl in 0.1 M sodium acetate buffer, pH 4.0, and 20 mM potassium phosphate buffer, pH 7.0.

**Purification of Thiol Proteinase Inhibitor**—Inhibitor was purified from rat liver essentially as described previously (9). Sequence examination showed that the inhibitor isolated on an active papain affinity column had undergone limited proteolysis (11).
thiocyanate-inactivated column was used in previous studies to prevent proteolysis of the inhibitor during its elution (9). For complete prevention of proteolysis, we used a column of carboxymethylated papain-Sepharose 4B in the present study. The purification steps included affinity chromatography on carboxymethylated papain, Sephadex G-75 chromatography, and DEAE-cellulose chromatography. The supernatant of 100 ml of liver extract was applied to the affinity column. At the G-75 step, two peaks of inhibitory activity were obtained (Fig. 1). The major fraction of lower molecular weight was dialyzed overnight at 4°C against 20 mM Tris-HCl, pH 7.5, and applied to a DEAE-cellulose (DE-52) column (1 × 18 cm) equilibrated with 20 mM Tris-HCl, pH 7.5. The column was washed with 0.2% Coomassie Brilliant Blue R-250 in 20 mM NaCl in 20 mM Tris-HCl, pH 7.5. Fractions with inhibitory activity against papain were pooled, concentrated, and stored at −20°C.

Reduction and Alkylation of Thiol Proteinase Inhibitor—The inhibitor was treated with 10 mM dithiothreitol in 0.1 mM Tris-HCl buffer, pH 8.0, for 20 min at room temperature in the absence and presence of 6 M guanidine hydrochloride and then with 18 mM iodoacetate or iodoacetamide for 20 min. The resulting solution was dialyzed for 24 h against five changes of 20 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, at 4°C. Incorporation of carboxymethyl groups was monitored by measuring S-carboxymethylcysteine after hydrolysis with 6 M HCl in vacuo.

Trituration of SH Groups of Thiol Proteinase Inhibitor with DTNB—The SH groups of thiol proteinase inhibitor were titrated by the method of Ellman (14). The reaction mixture contained 0.05 mM potassium phosphate buffer, pH 8.0, 0.1 mM DTNB and thiol proteinase inhibitor in a final volume of 1.0 ml.

Polyacrylamide Gel Electrophoresis—Analytical native polyacrylamide gel electrophoresis was carried out at pH 8.0 as described by Williams and Reisfeld (15). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli in 15% gel (16). Gels were stained with Coomassie Brilliant Blue R-250 in malton/maleic acid/water (25:7:68, v/v/v).

Preparation of [glycine-2-3H]GSSG—[glycine-2-3H]GSH was dissolved in 0.05 mM Tris-HCl (pH 8.0), and [3H]GSSG was prepared from it by the method of Usami et al. (17).

Incorporation of [3H]GSSG into Thiol Proteinase Inhibitor—TPI-2 was reduced with 2 mM dithiothreitol and incubated with [glycine-2-3H]GSSG in the presence of 20 mM unlabeled GSSG. The incubation mixture was then applied to a column of Sephadex G-25. The radioactive peak, coinciding with the inhibitory activity, was subjected to polyacrylamide gel electrophoresis without SDS. After electrophoresis the gel was cut into 2-mm sections, which were incubated with 20 mM Tris-HCl buffer, pH 7.5. The extracts were then dissolved in ACS-II (Amersham) and radioactivity was measured in a scintillation counter.

Cyanogen Bromide Digestion of [14C]Carboxymethylated Thiol Proteinase Inhibitor—TPI-2 was reduced and carboxymethylated with [14C]iodoacetate in the absence and presence of 6 mM guanidine hydrochloride. [14C]Carboxymethylated inhibitor at 1% protein in 70% formic acid was digested with 1% BrCN at 25°C for 5 h. The resulting BrCN peptides were dissolved in 7% formic acid and applied to a Sephadex G-25 column (1 × 45 cm) equilibrated with buffer containing formic acid, acetic acid, and water (25:5:788, v/v/v). Each fraction of eluate was neutralized and mixed with ACS-II, and radioactivity was measured in a scintillation counter.

Isolation of Glutathione Bound to Thiol Proteinase Inhibitor—TPI-2 was reduced with 2 mM dithiothreitol. After the pH of the solution was adjusted to 2 with HCl, it was applied to a column of Sephadex G-25 (1.0 × 50 cm) equilibrated with 20 mM HCl. SH groups of glutathione were detected with DTNB. The positive fractions, which were devoid of inhibitory activity, were collected. After the solution was lyophilized, the sample was hydrolyzed at 110°C for 24 h in 6 N HCl in vacuo. The HCl was removed under reduced pressure, and the residue was dialyzed in 0.5 ml of 20 mM HCl. Amino acid analyses were performed on a Hitachi Model 835-30 amino acid analyzer.

RESULTS

Isolation of Three Forms of Thiol Proteinase Inhibitor from Rat Liver—The thiol proteinase inhibitor was purified from rat liver on a carboxymethylated papain affinity column. Fig. 1A shows a typical elution pattern on Sephadex G-75. Two peaks of inhibitory activity (peak a and peak b) were obtained in positions corresponding to Mr = 24,000 and 12,000, respectively. On native polyacrylamide gel electrophoresis, peak a gave one protein band but peak b gave two bands (Fig. 1A, inset). The inhibitors of lower molecular weight in peak b gave two bands corresponding to Mr = 24,000 and 12,000, respectively. On native polyacrylamide gel electrophoresis, peak a and peak b gave two bands, corresponding to Mr = 24,000 and 12,000, respectively. On native polyacrylamide gel electrophoresis, peak a and peak b gave two bands, corresponding to Mr = 24,000 and 12,000, respectively.
corresponded to $M_r = 24,000$, indicating that the inhibitor in peak a is in a dimeric form (TPI-D) with a disulfide bridge.

Effect of Thiol Compounds on Interconversions of Different Forms of Inhibitor and Formation of a Complex between the Inhibitor and Cathepsin H—TP1-1 was incubated with or without 2 mM dithiothreitol at 25 °C for 10 min and then the mobility on nondenaturating polyacrylamide gel electrophoresis was examined. As shown in Fig. 3A, the mobility of TPI-1 was not affected (lane 2). The activity of TPI-1 was examined in the presence or absence of 2 mM dithiothreitol by testing its ability to form a complex with cathepsin H. Results showed that the complex was formed both in the presence (lane 4) and absence (lane 3) of dithiothreitol. In a similar experiment, TPI-2 was completely shifted to the position of TPI-1 when incubated with 2 mM dithiothreitol (B, lane 2). TPI-2 was inactive and did not form a complex with cathepsin H in the absence of dithiothreitol (B, lane 3). For formation of a complex, it was necessary to add 1.0 mM dithiothreitol to the reaction mixture (B, lane 4), with shift of TPI-2 to the position of TPI-1. For this effect, dithiothreitol could be replaced by reduced glutathione, 2-mercaptoethanol, or cysteine (not shown). Dithiothreitol was the most active agent, a concentration of 0.5 mM being sufficient for a complete shift of TPI-2 to the position of TPI-1. Disulfides, such as oxidized glutathione, cystine, and cystamine did not shift TPI-2. TPI-D was also moved to the position of TPI-1 by incubation with dithiothreitol (C, lane 2). TPI-D was inactive and did not form a complex with cathepsin H in the absence of dithiothreitol (C, lane 3), but was converted to an active form on addition of dithiothreitol (C, lane 4). Reaction mixtures containing the dimer form of the inhibitor, cathepsin H, and 2 mM dithiothreitol in 50 mM Tris-HCl, pH 7.0, were incubated at 25 °C for 10 min and then applied to a Sephadex G-75 column (1 × 80 cm) equilibrated with 50 mM Tris-HCl, pH 7.0. One major and one minor protein peak were observed in positions corresponding to $M_r = 37,000$ and 12,000, respectively (data not shown). Neither cathepsin H nor inhibitor activity was detected in the major peak, indicating that it was that of the complex, but inhibitor activity was detected in the minor peak. From its apparent molecular weight, the complex seems to be formed between cathepsin H and monomeric inhibitor, not the dimer inhibitor formed in the presence of dithiothreitol.

Cysteine Contents of the Three Forms of Inhibitor—To determine whether the sulfhydryl groups of the three forms of the inhibitor are involved in the interconversions and inhibitory activities of these forms, we measured the numbers of cysteine residues in the inhibitors. When TPI-1, TPI-2, and TPI-D were titrated with DTNB in the presence of 0.5% SDS, one SH group/mol of monomer was titrated in each form of the inhibitor. Next, the three forms of the inhibitor were reduced and carboxymethylated in the absence and presence of 6 M guanidine hydrochloride and their Cm-cysteine contents measured by amino acid analysis. As shown in Table I, there were no significant differences in the amino acid compositions of the three forms of the inhibitor. The Cm-cysteine contents of Cm-TPI-1 in the absence and presence of the denaturant were 0.2 and 1.1 residues/mol, respectively. But in Cm-TPI-2 and Cm-TPI-D about one Cm-cysteine residue/mol of monomer was found in the absence of guanidine hydrochloride and about two residues/monomer inhibitor in the presence of the denaturant. These results suggest that one cysteiny1 residue exposed on the surface of TPI-2 is carboxymethylated after reduction and that the other cysteinyl residue of TPI-2 is buried in the molecule because it was carboxymethylated only in the presence of guanidine hydrochloride. Results also indicated that one cysteine residue on the surface of the TPI-1 molecule was barely reactive even after treatment with dithiothreitol.

Electrophoretic Properties and Inhibitor Activities of Car-

![Fig. 2. SDS-polyacrylamide gel electrophoresis of the three forms of thiol proteinase inhibitor. Lane 1, TPI-1 (5 pg); lane 2, TPI-2 (5 pg); lane 3, a mixture of TPI-1 (4 pg) and TPI-2 (4 pg); lane 4, a mixture of TPI-1 (5 pg) and TPI-D (5 pg); lanes 5, 6, and 7, TPI-D (10 pg). Electrophoresis was carried out in the presence (lanes 1–5) and absence (lanes 6 and 7) of 5% mercaptoethanol. The sample in lane 7 had been incubated with 50 mM iodoacetate for 20 min at room temperature before electrophoresis.](image)

![Fig. 3. Effect of dithiothreitol on the interconversion of three forms of thiol proteinase inhibitor and on formation of a complex with cathepsin H. TPI-1 (A), TPI-2 (B), and TPI-D (C) were each incubated in 0.05 M Tris-HCl buffer, pH 7.5, for 10 min with no other addition (lane 1), 2 mM dithiothreitol (lane 2), cathepsin H (lane 3), or cathepsin H plus 1 mM dithiothreitol (lane 4) and then were subjected to polyacrylamide gel electrophoresis without SDS.](image)
phoresis without SDS, TPI-1, carboxymethylated in the presence of guanidine hydrochloride (denatured Cm-TPI-2), although the former moved faster on the gel than the latter.

Band of untreated TPI-2 (Fig. 4A, lane 2); the presence of dithiothreitol did not affect its mobility (Fig. 4A, lane 3). Denatured Cm-TPI-1 could form a complex with cathepsin H (Fig. 4A, lane 5), like untreated TPI-2 (Fig. 4A, lane 4), although the former moved faster on the gel than the latter.

There was no difference in the electrophoretic mobilities of untreated TPI-2 and Cm-TPI-2 (Fig. 4B, lanes 1 and 2), but Cm-TPI-2 did not shift to the position of TPI-1 on addition of diithiothreitol (Fig. 4B, lane 5). Denatured Cm-TPI-2 migrated faster than untreated TPI-2 or Cm-TPI-2 on gel electrophoresis (Fig. 4B, lane 3) and its electrophoretic mobility was not affected by diithiothreitol (Fig. 4B, lane 6). Cm-TPI-2 or denatured Cm-TPI-2 did not form a complex with cathepsin H irrespective of the presence of diithiothreitol (Fig. 4B, lanes 8 and 9). Cm-TPI-D and denatured Cm-TPI-D showed the same electrophoretic mobilities as Cm-TPI-2 and denatured Cm-TPI-2, respectively (Fig. 4C), and were inactive even in the presence of diithiothreitol.

These results indicate that carboxymethylation of the inhibitor with iodoacetic acid caused an increase in its negative charge.

**Interconversion of TPI-2 and TPI-D**—When TPI-D was incubated with 20 mM GSSG after reduction with 1 mM diithiothreitol, it migrated in the same position as TPI-2 on non-denaturing polyacrylamide gel electrophoresis. Conversely, extensive dialysis of TPI-2 after reduction with 2 mM diithiothreitol resulted in conversion of TPI-2 to TPI-D (not shown).

**Relation of Electrophoretic Mobility of TPI-2 to Inhibitor Activities**—TPI-2 was reacted with iodoacacetamide after reduction with diithiothreitol in the absence of 6 M guanidine hydrochloride. Carboxamidomethylated TPI-2 migrated in the same position as TPI-1 (Fig. 5, lane 2). It was active and formed a complex with cathepsin H (Fig. 5, lane 4). These results indicate that the inhibitor activity is closely related to the charge of the cysteine residue on the surface of TPI-2. To confirm this conclusion, we reacted TPI-2 with 20 mM cystamine (Fig. 6, lane 2), 20 mM homocystine (Fig. 6, lane 3) and 20 mM oxidized glutathione (Fig. 6, lane 4) after reduction with 1 mM diithiothreitol and examined the change in electrophoretic mobility by polyacrylamide gel electrophoresis without SDS. The electrophoretic mobility of the inhibitor differed depending on the charge of the disulfides. Results indicated that the free sulfhydryl group on TPI-2 produced by reduction reacted with the various added reagents to form mixed disulfides. TPI-2, incubated with cystamine and homocystine, was active and formed a complex with cathepsin H, but like TPI-

### Table 1

| Amino acid       | TPI-1 | TPI-2 | TPI-D | Deduced from sequence |
|------------------|-------|-------|-------|-----------------------|
|                   | Cm\(^\text{a}\) | D-Cm\(^\text{a}\) | Cm | D-Cm |
| Aspartic acid     | 8.1   | 8.2   | 7.9   | 7.9   | 7.9   | 7.9   | 8    |
| Threonine         | 7.6   | 7.5   | 7.7   | 7.6   | 7.7   | 7.6   | 8    |
| Serine            | 4.8   | 4.9   | 4.7   | 4.6   | 4.7   | 4.5   | 5    |
| Glutamic acid     | 15.5  | 15.4  | 15.4  | 15.4  | 15.3  | 15    |
| Glycine           | 3.3   | 3.4   | 3.1   | 3.2   | 3.1   | 3.1   | 3    |
| Alanine           | 7.1   | 7.2   | 7.1   | 7.1   | 7.0   | 7    |
| Valine            | 6.6   | 6.3   | 6.4   | 6.3   | 6.0   | 6    |
| Half-cystine      | 0.2\(^\text{b}\) | 1.1\(^\text{b}\) | 0.9\(^\text{b}\) | 1.7\(^\text{b}\) | 0.9\(^\text{b}\) | 1.7\(^\text{b}\) | 2    |
| Methionine        | 2.5   | 2.5   | 2.9   | 2.9   | 2.9   | 2.9   | 3    |
| Isoleucine        | 2.8   | 2.7   | 2.8   | 2.8   | 2.8   | 2.8   | 3    |
| Leucine           | 5.9   | 5.8   | 5.9   | 5.9   | 5.9   | 5.9   | 6    |
| Tyrosine          | 2.1   | 1.8   | 2.0   | 2.0   | 2.1   | 2.1   | 2    |
| Phenylalanine     | 6.8   | 6.7   | 6.7   | 6.7   | 6.8   | 6.8   | 7    |
| Lysine            | 10.4  | 10.3  | 9.9   | 10.1  | 10.1  | 10.2  | 10   |
| Histidine         | 3.0   | 3.0   | 2.9   | 2.9   | 2.9   | 2.9   | 3    |
| Arginine          | 3.3   | 3.2   | 3.1   | 3.1   | 3.3   | 3.2   | 3    |
| Proline           | 5.1   | 5.0   | 5.2   | 5.2   | 5.2   | 5.3   | 5    |

\(^\text{a}\) Carboxymethylated in the absence of 6 M guanidine hydrochloride.

\(^\text{b}\) Carboxymethylated in the presence of 6 M guanidine hydrochloride.

\(^\text{c}\) Cited from Ref. 11.

\(^\text{d}\) Values after 24-h hydrolysis.

\(^\text{e}\) Determined as S-carboxymethylcysteine.

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**Fig. 4.** The role of SH groups in the three forms of thiol proteinase inhibitors in their interconversion and activities. The three forms of the inhibitor were carboxymethylated in the absence or presence of 6 M guanidine hydrochloride as described under "Experimental Procedures." A, lane 1, TPI-1; lane 2, denatured Cm-TPI-1; lane 3, denatured Cm-TPI-1 plus 2 mM diithiothreitol; lane 4, TPI-1 plus cathepsin H; lane 5, denatured Cm-TPI-1 plus cathepsin H, B, lane 1, TPI-2; lane 2, Cm-TPI-2; lane 3, denatured Cm-TPI-2; lane 4, TPI-2 plus 2 mM diithiothreitol; lane 5, Cm-TPI-2 plus 2 mM diithiothreitol; lane 6, denatured Cm-TPI-2 plus 2 mM diithiothreitol; lane 7, TPI-2 plus cathepsin H in the presence of 1 mM diithiothreitol; lane 8, Cm-TPI-2 plus cathepsin H in the presence of 1 mM diithiothreitol; lane 9, denatured Cm-TPI-2 plus cathepsin H in the presence of 1 mM diithiothreitol. C, the same as for B but with TPI-D instead of TPI-2. Inhibitor (8 µg) and/or cathepsin H (16 µg) was subjected to non-denaturing polyacrylamide gel electrophoresis.
Three Forms of Thiol Proteinase Inhibitor from Rat Liver

FIG. 5. Effect of carboxamidomethylation of TPI-2 on formation of a complex. TPI-2 was carboxamidomethylated as described under "Experimental Procedures." Lane 1, TPI-2 (8 µg); lane 2, carboxamidomethylated TPI-2 (8 µg); lane 3, cathepsin H (16 µg); lane 4, carboxamidomethylated TPI-2 (8 µg) plus cathepsin H (16 µg). Samples were incubated in 20 µl of 0.05 M Tris-HCl, pH 7.5, at 25 °C for 5 min and then subjected to nondenaturing 7% polyacrylamide gel electrophoresis.

FIG. 6. Disulfide exchange between protein sulphydryl groups and disulfides. TPI-2 was reduced in 0.05 M Tris-HCl buffer, pH 8.0, by treatment with 1 mM dithiothreitol for 20 min at 25 °C, and then incubated with 20 mM cysteamine, homocystine, or GSSG. Samples were dialyzed against 0.05 M Tris-HCl, pH 7.5, overnight before electrophoresis. Lane 1, TPI-2 (8 µg); lane 2, TPI-2 (8 µg) plus cysteamine; lane 3, TPI-2 (8 µg) plus homocystine; lane 4, TPI-2 (8 µg) plus GSSG; lane 5, TPI-2 (8 µg) plus cysteamine plus cathepsin H (16 µg); lane 6, TPI-2 (8 µg) plus homocystine plus cathepsin H (16 µg); lane 7, TPI-2 (8 µg) plus GSSG plus cathepsin H (16 µg); lane 8, cathepsin H (16 µg).

2, the inhibitor incubated with oxidized glutathione was inactive in the absence of dithiothreitol (Fig. 6, lanes 5, 6, and 7).

The formation of a mixed disulfide between TPI-2 and glutathione was also examined by measuring incorporation of radioactivity into the protein after incubation with 3H-oxidized glutathione. After 20-min incubation of reduced TPI-2 with GSSG containing [3H]GSSG, the incubation mixture was subjected to gel filtration on a Sephadex G-25 column (1 × 45 cm), and the radioactivity and inhibitory activity of fractions of the eluate were measured. The peak position of the inhibitory activity measured in the presence of 4 mM cysteine coincided with the first peak of radioactivity. The second peak of radioactivity was that of remaining GSSG, as demonstrated by assay of GSH after reduction. When the fractions containing the inhibitor were pooled, concentrated, and subjected to polyacrylamide gel electrophoresis under nondenaturating conditions, radioactivity was obtained in a protein band that migrated in the same position as TPI-2 on a parallel gel (Fig. 7A). When 2 mM dithiothreitol was included in the incubation mixture, the radioactivity was recovered not in the protein but in the position of the marker dye, corresponding to authentic GSH. On addition of dithiothreitol, the inhibitor was recovered in the same position as TPI-1 (Fig. 7B). Thus, after reduction of the mixed disulfide of TPI-2, GSSG reacted with the free thiol group on TPI-2 to form a mixed disulfide and the inhibitory activity was lost again. Addition of reducing agents such as dithiothreitol cleaved the mixed disulfide and caused the change in electrophoretic mobility and recovery of inhibitor activity.

Digestion of TPI with Cyanogen Bromide—The inhibitor consists of 98 amino acid residues and its two cysteine residues are residues 3 and 64 (11). It seemed interesting to determine which cysteine residue was involved in formation of the mixed disulfide. On cyanogen bromide digestion of the inhibitor, three major fragments are obtained (residues 11-98, residues 3-10, and an N-terminal fragment). The amino acid sequence of rat liver TPI (11) is: acetyl-MMCGAPSATMPATTETQ EIADKVKSEEEKANQKFDFVKSAIFRQQVVGATNFI

64

KVDVGEVK CVHLRFEPLPHEKPLTLSSYQTDEK

88

HDELTYPF Thus, when a digest of the inhibitor with cyanogen bromide is applied to a Sephadex G-25 column, a large fragment containing cysteine 64 and a small fragment containing cysteine 3 should separate. TPI-2 was reduced and
From the following observations it appears that TPI-2 is most likely to be a mixed disulfide with glutathione. (a) Incubation of the inhibitor with a reducing agent caused a change in electrophoretic mobility (Fig. 3), which was reversed by reduction and carboxymethylation (Fig. 4). (b) When TPI-2 was reacted with low-molecular-weight disulfides after reduction, its mobility on polyacrylamide gel electrophoresis depended on the charge of the disulfide (Fig. 6), indicating disulfide exchange between the protein sulfhydryl group and the disulfide. Incubation with GSSG did not affect the mobility of TPI-2. (c) Reaction of TPI-2 with radioactive disulfide in the presence of a reducing agent led to incorporation of the isotope into the inhibitor, and this radioactivity in the protein was lost on subsequent treatment with reducing agent (Fig. 7). Measurement of carboxymethylcysteine in TPI-2 after alkylation in the absence and presence of guanidine hydrochloride showed that one cysteine residue is on the surface of the molecule and the other is buried (Table I). The sulfhydryl group on the surface of the molecule can interact with disulfide and is related to the formation of a mixed disulfide. The fact that Cys-3 is at the surface was demonstrated by analyzing CNBr digests of 14C-labeled Cm-TPI-2 (Fig. 8). (d) When isolated TPI-2 was subjected to amino acid analysis without reduction, it contained 0.4, 0.9, and 0.7 residues of cysteine, glycine, and glutamic acid, respectively, more than reduced and carboxymethylated TPI-2 (data not shown), and in fact, glutathione was isolated from TPI-2 after reduction.

Isolated TPI-2 cannot form a complex with cathepsin H in the absence of a thiourea compound, and this seems to be related to the charge of the protein mixed disulfide. The inhibitor became inactive on disulfide exchange with a negatively charged disulfide (GSSG), but reaction with cystamine or homocysteine restored its activity (Fig. 6). This conclusion is supported by the observation that carboxymethylated TPI-2 was inactive, but that the carboxamidomethylated inhibitor was active (Fig. 5). These results indicate that a free sulfhydryl group on Cys-3 is not essential for formation of a complex with cathepsin H, and that introduction of a negative charge at the cysteine residue interferes with complex formation.

TPI-D was also inactive and the reductive cleavage of its intermolecular disulfide bond restored activity (Fig. 3). TPI-D and TPI-2 could be interconverted in vitro, but conversion of TPI-D or TPI-2 to TPI-1 was unsuccessful. Cys-3, the surface sulfhydryl group in TPI-1, is not free, and not reduced or carboxymethylated (Fig. 3, Table I). It may have some special structure, the nature of which is unknown, which is resistant to reduction, or the amino-terminal sequence containing Cys-3 may not be present in TPI-1. The second possibility seems very unlikely because the content of methionine, located at residues 1, 2, and 10 (11), is the same in TPI-1, TPI-2, and TPI-D (Table I).

Both cysteine Cys-3 and Cys-64 of the thiol proteinase inhibitor are probably free immediately after synthesis. Three forms of the thiol proteinase inhibitor are found after papain-affinity chromatography. Removal of low-molecular-weight thiol compounds may be a major factor in formation of these three forms. The very low pH used in elution of the inhibitor from the column may also promote their formation. However, the fact that GSH is isolated from TPI-2 after reduction suggests the formation of TPI-2 in vivo.

The activities of many enzymes (17, 20-25) have been suggested to be regulated by the formation of protein mixed disulfides, which has a dramatic effect on their catalytic activities. The present results indicate that the thiol proteinase inhibitor from rat liver is a typical example of this type of protein. Since it is found mainly in the soluble fraction (9),
the cytosolic level of GSH and the GSH/GSSG ratio may influence the level of the active thiol proteinase inhibitor in the liver. In fact, mixed disulfides between protein and glutathione or cysteine are present in various mammalian tissues (26), and the amounts of these modified proteins may vary diurnally (27) or in response to the feeding cycle of the animal (28). However, the physiological significance of the inhibitory activity by formation of mixed disulfides and by formation of a dimer through an intermolecular disulfide bridge awaits further investigation.

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