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GENERAL SPECIFICITY OF CYTOPLASMIC THIOLTRANSFERASE (THIOL:DISULFIDE OXIDOREDUCTASE) FROM RAT LIVER FOR THIOL AND DISULFIDE SUBSTRATES

KENT AXELSSON and BENGT MANNERVIK

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

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Summary

The substrate specificity of thioltransferase (thiol:disulfide oxidoreductase) purified from rat liver cytosol (Axelsson, K., Eriksson, S. and Mannervik, B. (1978) Biochemistry 17, 2978—2984) has been investigated. Various low-molecular-weight thiols were found to be substrates in the scission of sulfur-sulfur bonds in cystamine and glutathione disulfide as well as in mixed disulfides of proteins and glutathione. Protein sulfhydryl groups could also serve as thiol substrates in enzyme-catalyzed thiol-disulfide interchange. Thioltransferase also catalyzed the reduction of sulfur-sulfur bonds in disulfide-containing proteins by glutathione provided that the proteins had been denatured by proteolysis or urea treatment. It is proposed that thioltransferase may have a role in the intracellular protein degradation by cleavage of disulfide bonds. The finding that thioltransferase catalyzes the reversible formation of mixed disulfides of glutathione and the bulk of proteins in rat liver cytosol is relevant for the catalysis of changes in the 'glutathione status' in the cell. In conclusion, the results of the present investigation show that cytoplasmic thioltransferase has a very broad substrate specificity, which implies that the enzyme may participate in a multitude of thiol-disulfide interchange reactions.

Introduction

Cytoplasmic thioltransferase (thiol:disulfide oxidoreductase) has been purified from rat liver and characterized by Axelsson et al. [1]. The enzyme is a small-molecular-weight glycoprotein with a high isoelectric point [1] and is distinct from the membrane-bound enzymes catalyzing thiol-disulfide interchange [2—5]. The name of thioltransferase was introduced as a replacement of 'transhydrogenase' previously used for the enzymes catalyzing thiol-disulfide interchange [6]. The cytoplasmic thioltransferase has been found to catalyze the reduction of sulfur-sulfur bonds in low-molecular weight disulfides and thiosulfate esters by use of reduced glutathione (GSH) as a reductant [1,7—10]. It has been proposed that thioltransferase in concert with glutathione reductase catalyzes a major route of reduction of disulfides and thiosulfate esters [9,11]. Furthermore, scission by GSH of disulfide bonds in some protein mixed disulfides has been found to be catalyzed by the purified thioltransferase [12]. A good model substrate for the latter reaction was the mixed disulfide of egg-white lysozyme and GSH [13]. Also a crude preparation of cytoplasmic proteins from rat liver, which had been reacted with glutathione disulfide (GSSG) to make mixed disulfides with protein sulfhydryl groups, was a substrate for the cytoplasmic thioltransferase [11,14,15]. The present investigation was undertaken to further explore the substrate specificity of the enzyme with respect to thiol as well as disulfide substrate. Some of the results have been presented [11,15].

Materials and Methods

Chemicals. Trypsin, α-chymotrypsin, pronase P (*Streptomyces griseus* protease, type VI), bovine pancreatic ribonuclease, bovine serum albumin, ovalbumin, reduced glutathione (GSH), dithiothreitol, cysteamine, L-cysteine, penicillamine, thioglycollic acid, and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); egg-white lysozyme from Serva Feinbiochemica GmbH and Co. (Heidelberg, F.R.G.); yeast glutathione reductase, glutathione disulfide (GSSG), and coenzyme A from Boehringer Mannheim (Mannheim, F.R.G.). L-[*Glycine*-2-^{3}H]glutathione, 250 Ci/mol, Protosol, and Aquasol were obtained from New England Nuclear Co. (Dreieich, F.R.G.). Labelled GSSG was prepared by bubbling air for 15 h through a solution of labelled GSH at pH 8—10. The oxidized product was then mixed with an appropriate amount of unlabelled GSSG before use. [^{35}S]Cystamine (100 Ci/mol) was obtained from The Radiochemical Centre Ltd. (Amersham, England). Other substances were standard analytical grade chemicals.

Cytoplasmic thioltransferase from rat liver was purified and assayed as previously described [1]; activity units (in μmol/min) are based on measurements with *S*-sulfocysteine and GSH as substrates [1].

Preparation of reduced protein fractions from rat liver

A postmicrosomal supernatant fraction of rat liver, homogenized in 0.25 M sucrose, was chromatographed on a bed of Sephadex G-75 equilibrated with 10 mM sodium phosphate (pH 6.1), containing 1 mM EDTA, in order to remove endogenous thioltransferase and low-molecular weight components (see Refs. 1 and 12). The bulk of the protein fraction was adjusted to pH 8.2 with cold 0.2 M NaOH and treated with 0.2 μmol dithiothreitol per mg protein to reduce disulfide bonds (e.g. of mixed disulfides with GSH; see Refs. 16—18). Excess reagents were removed by passing the reduced protein fraction over a Sephadex G-25 column. The protein fraction was concentrated to 60 mg/ml by ultrafiltration on an Amicon PM 10 membrane. The concentrated stock solution contained 0.1 μmol sulfhydryl groups per mg protein as determined with 5,5′-dithiobis(2-nitrobenzoate) [19]. The stock solution was stored at −20°C.

A microsomal fraction was prepared as described by Ernster et al. [20]. The microsomes were treated at pH 7.5 for 24 h at 4°C with 2 mM dithiothreitol (0.2 μmol per mg of microsomal protein). Excess reagents were removed by dialysis. The microsomes contained 6 nmol of titrable sulfhydryl groups per mg protein after this treatment.

Preparation of mixed disulfides of cytosolic proteins and glutathione

The reduced protein fraction from rat liver cytosol (see above) was added dropwise to a solution of radioactively labelled GSSG (2 Ci/mol) at pH 8.5 and allowed to react at 0°C. The initial concentration of GSSG was 2.5 mM and the total amount of protein added had a sulfhydryl titer corresponding to a quarter of the molar amount of GSSG. After 2 h reaction time the mixture was chromatographed on a Sephadex G-50 column and the labelled protein concentrated by ultrafiltration (on an Amicon PM 10 membrane). The final product contained 0.18 μmol of glutathione sulfenyl groups per mg of protein. The protein-bound glutathione moieties were determined in a sample precipitated by trichloroacetic acid (see under 'Assay of formation of protein mixed disulfides') and, thus, probably does not include non-covalently bound GSSG. Therefore, the difference in sulfhydryl groups determined with 5,5′-dithiobis-(2-nitrobenzoate) (0.1 μmol/mg) and the bound glutathione residues (0.18 μmol/mg) appears to reflect differences in availability of sulfhydryl groups in the two reactions.

Procedure to measure thiol-disulfide interchange of low-molecular-weight thiols and disulfides

The reactions between radioactively labelled GSSG and various thiols were tested in 0.25 ml of 0.12 M sodium phosphate buffer (pH 6.5) containing 0.5 mM EDTA at 30°C. GSSG (1.8 Ci/mol) was used in at 6 mM and the thiols at 2 mM concentrations. Thioltransferase (0.075 unit) was included when the enzymatic reaction was measured. The assay conditions for measuring thiol-disulfide interchange with labelled cystamine (6 mM, 0.4 Ci/mol) and various thiols (2 mM) were the same. The pH value was kept low (below the optimum for thioltransferase) in the system in order to reduce the rate of the spontaneous reaction, which otherwise is too rapid with the concentrations required in the system to optimize the subsequent separation and detection of reactants. 5-μl aliquots were taken from the reaction system at different time points and spotted on a Whatman No. 1 filter paper 18.5 × 39 cm. The paper had been cut into 1-cm wide parallel lanes separated by 0.5 cm wide and 24 cm long slots. Each aliquot was spotted on a lane 4 cm from the end. The place of application had previously been soaked with 10 μl of 1 M *N*-ethylmaleimide dissolved in 99.5% ethanol and dried. By reaction with this reagent the thiol-disulfide interchange is stopped and oxidation of the thiols to disulfides prevented. Paper electrophoresis was carried out for 180 min at 350 V with 5 M acetic acid as buffer. The electrophoretograms were developed with ninhydrin. The spots on the paper were cut out, placed in scintillation vials and decolorized by addition of 100 μl formic acid followed by 100 μl H_2O_2. After decolorization and evaporation of reagents 5 ml Aquasol were added to each vial and the radioactivity counted in a Beckman model LS 100 liquid scintillation spectrometer.

Assay of the reduction by various thiols of mixed disulfides of cytosolic proteins and glutathione

The reaction system of 0.2 ml contained: mixed disulfides of proteins and labelled glutathione (1.25 mg/ml) prepared as described above, a low-molecular-weight thiol (0.5 mM), purified thioltransferase (0.375 unit/ml), sodium phosphate buffer (pH 7.5, 0.12 M), and 1 mM EDTA. For measurement of the spontaneous reaction the thioltransferase was omitted. The reaction was started by addition of thiol and was stopped by addition of 0.8 ml of ice-cold 15% trichloroacetic acid. After centrifugation, the supernatant fraction was mixed with 10 ml Aquasol and the radioactivity counted. By this count a direct measure of the amount of GSH liberated by thiol-disulfide exchange was obtained. The reactions were also followed by determination of the radioactivity in the pelleted material; this was measured as described below for the formation of protein mixed disulfides.

Assay of formation of protein mixed disulfides

The reaction of the reduced proteins in the cytosol fraction from rat liver (see above) was measured in an assay system containing in 0.4 ml total volume: 15 mg/ml reduced protein, 1.5 mM labelled GSSG or cystamine, 0.5 mM EDTA, and pH 7.5, 0.1 M sodium phosphate buffer. Thioltransferase (0.1 unit) was also included when the enzymatic reaction was studied. The reaction was stopped by addition of 0.6 ml ice-cold 15% (w/v) trichloroacetic acid. An addition of 100 μl of 20 mM GSSG (or cystamine) was then made to prevent unspecific binding of radioactivity. The samples were centrifuged, the supernatant fractions discarded, and the pellets dissolved in 0.8 ml Protosol at 22°C overnight. The dissolved pellets were mixed with 10 ml Aquasol and the radioactivity measured by liquid scintillation counting.

The reaction of 'reduced' microsomes with labelled cystamine was determined in the assay system described above. The concentration of microsomal protein in the system was 2.5 mg/ml.

Assay of disulfide reduction in denatured proteins

Disulfide-containing proteins were pretreated at 30°C with a mixture of trypsin, chymotrypsin, and pronase P in 40 mM Tris-HCl buffer (pH 8.1) containing 10 mM $CaCl_2$. The enzymes were each added to a final concentration of 0.4 μM; the actual concentration of the substrate proteins are given in Table III. The proteins were incubated with the proteases for 20 h. When urea was used as denaturant, the substrate proteins were incubated for 20 min at 30°C with 8 M urea in 0.1 M sodium phosphate buffer (pH 7.5). Aliquots (200 μl) of pretreated proteins were then taken for measurements of the rate of GSH-dependent disulfide reduction in a system containing 0.5 mM GSH, 0.1 mM NADPH, 2 units of glutathione reductase, 0.1 unit of purified thioltransferase and 0.13 M Tris-HCl buffer (pH 8.1). The rate of the spontaneous reaction was measured in the absence of thioltransferase.

Fitting of progress curves

For determination of initial rates of progress curves the data corresponding to different time points were fitted by a third degree polynomial

$$y = a_1 t + a_2 t^2 + a_3 t^3$$

where y is the concentration of product of the reaction, t is time, and a_i (i = 1—3) are parameters of the regression. The initial rate of the reaction is $(\mathrm{d}y/\mathrm{d}t)_{t=0} = a_1$. The polynomial was fitted to the experimental data by the least-squares method. The results were evaluated by use of a series of goodness-of-fit criteria (cf. Refs. 21 and 22) and all regressions presented were considered as satisfactory.

Results

Reduction of low-molecular-weight disulfides

Previous investigations [1] have shown that purified cytoplasmic thioltransferase catalyzes the thiol-disulfide interchange of GSH and disulfides (or thiosulfate esters). These reactions were monitored spectrophotometrically by coupling to the glutathione-reductase-catalyzed oxidation of NADPH by GSSG. This coupled system requires that the thiol in the interchange reaction is GSH. In order to investigate the thiol-specificity of the thioltransferase it was therefore necessary to use a direct method for measuring interchange reactions. Paper electrophoresis has previously been used to measure spontaneous [23] as well as enzymatic [24] thiol-disulfide interchange. (For a discussion of alternative assay methods for thioltransferase, see Ref. 25.) Thus, thiol-disulfide interchange was studied by an electrophoretic method using radioactively labelled cystamine and GSSG as disulfide substrates and various thiols.

Fig. 1 shows typical progress curves of the spontaneous and thioltransferase-catalyzed reactions of coenzyme A and cystamine. Only the reactants containing radioactive label (cystamine, cysteamine, and the mixed disulfide of coenzyme A and cysteamine) were determined. It is evident that a significant enzymatic effect can be demonstrated on the disappearance of cystamine. However, the effect of thioltransferase on the appearance of the mixed disulfide or cysteamine is not clear. The explanation for this result is probably that the secondary reaction (Eqn. 2) in the set of interlinked reactions

$$\mathrm{C{-}SS{-}C^{*} + CoA{-}SH} \begin{array}{ll} \nearrow \mathrm{C{-}SS{-}CoA + {}^{*}C{-}SH} & \qquad (1a) \\ \searrow \mathrm{{}^{*}C{-}SS{-}CoA + C{-}SH} & \qquad (1b) \end{array}$$

$$\mathrm{{}^{*}C{-}SS{-}CoA + CoA{-}SH \rightarrow CoA{-}SS{-}CoA + {}^{*}C{-}SH} \qquad (2)$$

(where C-SS-C, C-SH, CoA-SH are cystamine, cysteamine, and coenzyme A, respectively, and C-SS-CoA the mixed disulfide) is proportionally more rapid in the presence of enzyme than the primary reactions (Eqns. 1a and b). Differential effects on such interlinked reactions have previously been observed when cystine and labelled GSH were used as substrates [6].

The conditions of the thiol-disulfide reactions were difficult to optimize because of the rapid spontaneous reaction and the time required for sampling

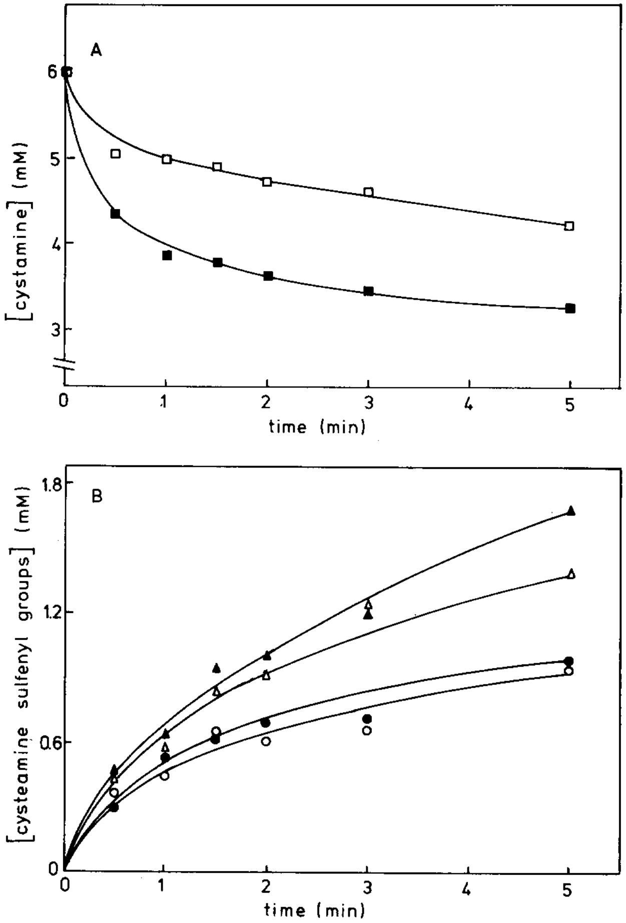

Fig. 1. Progress curves for thiol-disulfide interchange of radioactively labelled cystamine and coenzyme A. The reaction was followed at pH 6.5 and 30°C by measuring the concentration of (A) labelled cystamine (■, □) and the concentrations of (B) labelled products: mixed disulfide of cysteamine and coenzyme A (●, ○), and cysteamine (▲, △). The filled symbols refer to measurements carried out in the presence of thioltransferase.

from the reaction mixture. The pH optimum of thioltransferase is about 7.5 (for *S*-sulfoglutathione and GSH as substrates; Ref. 1), but at this pH value the spontaneous reaction is inconveniently rapid for the electrophoretic assay. As a compromise, pH 6.5 was used, in spite of the fact that the enzymatic activity is relatively low at this pH value. Experiments were also carried out at pH 6.0, 6.3 and 7.5. Table I shows the results of the investigations involving labelled cystamine and GSSG and various thiols evaluated in qualitative terms. It was found that the enzyme had a significant effect on the disappearance of cystamine and GSSG with all the thiols tested. The thiols which most clearly demonstrated the enzymatic catalysis were GSH and coenzyme A. Most reactions involving different thiols and cystamine showed a significant effect of thioltransferase on the formation of the corresponding mixed disulfides. On the other hand, only the reaction involving cysteamine showed an enzymatic increase of mixed disulfide when GSSG served as disulfide substrate. These

TABLE I

EFFECT OF THIOLTRANSFERASE ON THIOL-DISULFIDE INTERCHANGE

Different thiols (RSH) were tested as substrates for the thioltransferase-catalyzed thiol-disulfide interchange of radioactively labelled glutathione disulfide (GSSG) and cystamine (CSSC) (cf. Eqn. 1 and 2). Progress curves were determined by the electrophoretic method described in the text. Fig. 1 shows a typical experiment. The influence of the thioltransferase was evaluated qualitatively for each pair of substrates: ++, a major effect; +, a significant effect; 0, no measurable effect.

Thiol	Effect of thioltransferase					
	Reactions of glutathione disulfide			Reactions of cystamine		
	GSSG decrease	GSSR formation	GSH formation	CSSC decrease	CSSR formation	CSH formation
Coenzyme A	++	0	+	++	0	+
Cysteamine	+	+	+	+		+
L-Cysteine	+	0	+	+	+	0
Glutathione	++		+	++	+	+
Penicillamine	+	0	+	+	++	0

results indicate that the mixed disulfides formed from GSSG are good substrates and, therefore, rapidly transformed by the secondary reaction (cf. Eqn. 2). The mixed disulfides obtained from cystamine, on the other hand, appear to be less good substrates and, thus, some of them accumulate transiently. Previous investigations showed that k_{cat} values were similar for various substrates, but that the apparent K_m values ranged from 7—1700 μM [1]. Therefore, the mixed disulfides of cysteamine are probably less good substrates owing to high K_m values rather than low k_{cat} values. In conclusion, the enzyme has a wide substrate specificity and can utilize other thiol substrates than GSH for reduction of sulfur-sulfur bonds in symmetrical as well as mixed disulfides.

Reduction of mixed disulfides of protein and GSH

A substantial fraction of the cellular glutathione appears to be bound as mixed disulfides of proteins in the cytosol [16—18,26,27]. Diurnal variations of the levels of GSH and mixed disulfides of proteins have been described [18,28], and it was important to investigate the role of cytoplasmic thioltransferase in the formation and decomposition of protein mixed disulfides. A crude protein fraction from rat liver cytosol was reacted with labelled GSSG to make a mixed disulfide. This reaction has previously been found to be catalyzed by thioltransferase, as has the reversal of the reaction [11,14,15]. The present investigation confirms these findings and extends them by the use of alternative thiols for the scission of the sulfur-sulfur bonds of the protein mixed disulfides.

Fig. 2 demonstrates typical progress curves of the liberation of GSH from the labelled protein-mixed disulfides by use of coenzyme A or GSH. The catalytic effect of thioltransferase is evident, even if most of it has been exerted before the first sample was taken from the reaction mixture. The latter statement is founded on the difference between the progress curves obtained in the absence and the presence of thioltransferase which is almost constant in the time range studied. Thus, the enzyme has a very high activity with the protein-

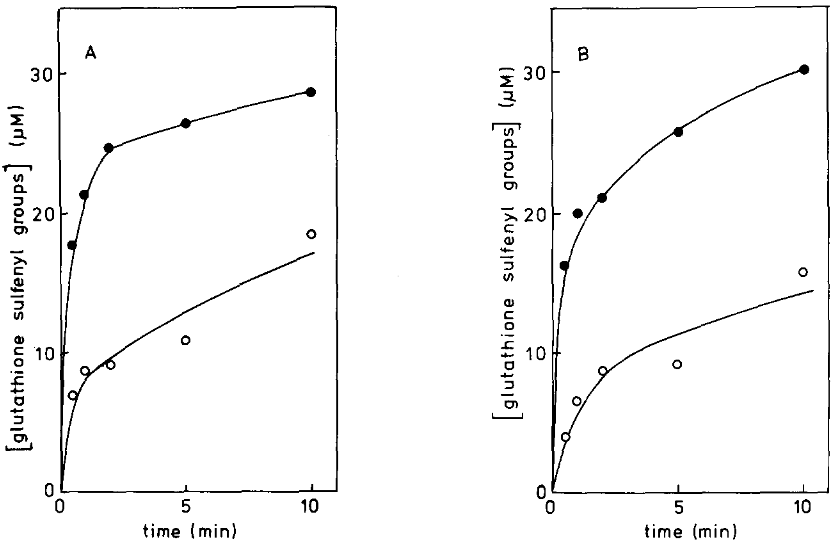

Fig. 2. Progress curves for the release of glutathione sulfenyl groups from mixed disulfides of cytoplasmic proteins and glutathione. The reaction system contained 1.25 mg/ml of protein mixed disulfides (0.18 μmol glutathione sulfenyl groups per mg) which were reacted at pH 7.5 and 30° C with 0.5 mM thiol in the presence (●) or absence (○) of thioltransferase (0.375 unit/ml). The thiol was in (A) glutathione and in (B) coenzyme A.

mixed disulfide resulting in an almost instantaneous reduction of the accessible disulfide bonds. The slow reaction proceeding after the first minute of reaction time is apparently not limited by the rate of thiol-disulfide interchange, but probably by conformational changes of the protein substrate which make additional disulfide bonds available for reaction.

In order to evaluate the first rapid phase of the reaction, the progress curves were fitted by a third degree polynomial passing through the origin in the corresponding diagram. The coefficient of the first degree term is equal to the initial rate of the reaction. Table II shows the results of the computer fits. All fits satisfied standard goodness-of-fit criteria [21,22]. It was found that, under the conditions investigated, no major differences existed in terms of activity of the various thiols. This statement applies to the spontaneous as well as the enzymatic reactions. The enzymatic velocities were in a range of about 6—15 μM/min for an enzyme concentration of 0.375 unit/ml. Recalculated on the basis of $M_r = 11\,000$ and a specific activity of 705 units per mg of pure thioltransferase [1] this range corresponds to a turnover of about 120—300 min^{-1}. The activity of thioltransferase versus the different thiols and the mixed disulfides of proteins and glutathione determined in the present study is lower than the molecular activity obtained with GSH and low-molecular-weight disulfides or thiosulfate esters (about 10^4 min^{-1}) [1]. However, the experiments have not been performed under identical conditions. In the present investigation 0.5 mM GSH was used instead of 4 mM in the earlier one [1]. This difference between the two GSH concentrations may result in a considerable difference in velocity. Since the protein mixed disulfides form a mixture of different substrates, the velocity should also be lower than expected for a pure disulfide

TABLE II

SCISSION OF MIXED DISULFIDES OF CYTOSOLIC PROTEINS AND GLUTATHIONE BY VARIOUS THIOLS

Mixed disulfides of cytosolic protein from rat liver and radioactively labelled glutathione were reacted with various thiols. The reactions were monitored by measurement of the release of labelled glutathione. The reaction system contained: 1.25 mg/ml of protein mixed disulfide (0.22 mM concentration expressed in glutathione sulfenyl groups), 0.5 mM thiol, 0.375 unit/ml thioltransferase (for the enzymatic reaction), 0.12 M sodium phosphate (pH 7.5), and 1 mM EDTA. The progress curves (cf. Fig. 2) were fitted by regression analysis to obtain initial rates. The enzymatic rates were obtained by subtraction of the spontaneous reaction rate and are given for the thioltransferase concentration in the assay system. The standard deviations (S.D.) of the enzymatic rates are calculated from the standard deviations for the corresponding rates in the presence and absence of thioltransferase.

Thiol	Initial rate ± S.D. (μM/min)	
	Spontaneous	Enzymatic
Coenzyme A	7.8 ± 0.5	13.8 ± 5.2
Cysteamine	5.4 ± 0.8	12.9 ± 3.0
L-Cysteine	9.4 ± 0.5	9.1 ± 4.6
Dithiothreitol	12.4 ± 0.8	10.8 ± 5.3
Glutathione	9.6 ± 2.2	15.3 ± 5.5
2-Mercaptoethanol	7.7 ± 0.8	14.9 ± 4.7
Penicillamine	6.2 ± 0.7	13.5 ± 2.9
Thioglycollate	6.6 ± 1.0	6.0 ± 3.8

substrate (cf. Ref. 29). In addition, it should be remarked that the values in Table II probably underestimate the rates somewhat because of the lack of experimental points in the initial part of the progress curves. Consequently, the molecular activity appears to be of the same order of magnitude for all substrates tested so far. Because of experimental difficulties, K_m values have not been determined in the present investigation, but previous results with the mixed disulfide of glutathione and egg-white lysozyme have shown a K_m value of 7 μM for this protein-disulfide substrate. The total concentration of glutathione sulfenyl groups in mixed disulfides of protein in rat liver has been determined as about 10 μmol/g liver (corresponding to an average concentration of about 10 mM) [28]. Thus, if many such protein mixed disulfudes have a low K_m value their reduction will not be limited by lack of saturation of the thioltransferase. All available data consequently support the proposal of mixed disulfides of proteins and GSH as an important class of natural substrates for the enzyme.

Formation of protein mixed disulfides

It has previously been shown that thioltransferase catalyzes the formation of mixed disulfides of GSSG and a reduced protein fraction from rat liver cytosol [11,14,15]. The enzymatic effect on this reaction was confirmed because of its possible importance in relation to the diurnal variations of the levels of GSH and mixed disulfides of protein and GSH [18,28] and the 'glutathione status' of the cell [30]. It was found that after 10 min reaction time 180 nmol of glutathione sulfenyl groups had been incorporated per mg of protein. The formation of mixed disulfides of cystamine and the crude protein fraction was also examined. However, in contrast to the reaction involving GSSG, only 1.1

nmol of mixed disulfide per mg protein was demonstrated under the same conditions. The latter result has relevance for the evaluation of the suggested function of cystamine in oxidizing sulfhydryl groups of nascent polypeptide chains [31], but this question requires further investigations before any conclusions can be drawn. It was also demonstrated that a microsome fraction from rat liver could incorporate cysteamine sulfenyl groups. A titer of 6 nmol of reactive sulfhydryl groups per mg of microsomal protein was found, and, after 10 min reaction with cystamine, 5.6 nmol of cysteamine sulfenyl groups per mg were bound to the trichloroacetic-acid-insoluble protein fraction.

Scission of protein disulfide bonds

In addition to the reactions involving protein mixed disulfides, it was also considered important to evaluate the possible role of thioltransferase in the scission of sulfur-sulfur bonds in disulfide-containing proteins. Four proteins were selected as model substrates: bovine serum albumin, egg-white lysozyme, bovine pancreatic ribonuclease, and ovalbumin. It was found that thioltransferase had a catalytic effect on the reduction of disulfide bonds in all cases in which a reaction could be detected. Untreated proteins were very resistant to reduction of their sulfur-sulfur bonds, as previously noted for the non-

TABLE III

EFFECT OF PROTEOLYSIS AND UREA DENATURATION ON GLUTATHIONE-DEPENDENT REDUCTION OF DISULFIDE BONDS IN PROTEINS

Proteins were pretreated with a mixture of proteases or 8 M urea for denaturation as described in the text. Aliquots (200 μl) were taken for determination of the rate of GSH- and thioltransferase-dependent reduction of disulfide bonds. In the assay system the aliquots were diluted 5-fold. The system contained 0.5 mM GSH and 0.1 unit/ml of thioltransferase. The velocities are initial rates; zero-order kinetics were maintained for 10—15% of the course of reaction. The enzymatic velocities have been recalculated to correspond to the apparent mean concentration of thioltransferase in rat liver cytosol. For this calculation a factor of 1.4 has been used to convert units per g wet weight to units per ml cytosol (cf. Table II and III in Ref. 34).

Protein	Pre-treatment	Protein concentration during pretreatment (μM)	Number of reducible disulfide bonds per protein molecule	Velocity (μM/min)	
				Spontaneous	Enzymatic
Albumin	Control	140	0.68	0.9	58
	Protease	140	10.6	2.2	662
	Control	1070	0.64	3.0	152
	Urea	1070	2.7	2.3	537
Lysozyme	Control	620	0.1	<0.04 *	<0.04 *
	Protease	620	1.1	1.3	104
	Urea	460	n.d. **	<0.04 *	<0.04 *
Ribonuclease	Control	580	0.1	1.6	16
	Protease	580	1.1	2.9	158
	Control	1770	0.03	2.2	28
	Urea	1770	0.07	2.7	104
Ovalbumin	Control	150	0.3	2.2	3.2
	Protease	150	2.1	2.9	41

* Below the detection limit in the assay (0.04 μM/min).

** Not detectable by the techniques used.

enzymatic reaction involving GSH [32,33], and the presence of thioltransferase did not increase the number of scissile bonds. However, if the proteins were pretreated with proteases or with 8 M urea, the number of accessible bonds increased significantly. Table III shows the results of the experiments. The effect of the thioltransferase has been recalculated to correspond to the expected enzyme concentration in the cytosol of hepatocytes. It is clear that for some substrates (e.g. pretreated albumin), the catalytic effect is quite substantial. It is possible that this activity of thioltransferase on denatured proteins may have a role in the intracellular degradation of proteins.

Discussion

The present investigation has extended the range of known substrates for the cytoplasmic thioltransferase from rat liver [1,14]. It had earlier been clearly established that the enzyme catalyzes the reaction between GSH and various glutathione sulfenyl derivatives, including mixed disulfides and *S*-sulfoglutathione [1]. The previous results also indicated that sulfur-sulfur bonds which were not linked to a glutathione moiety could be cleaved by GSH in two consecutive reactions:

$$\mathrm{RSSR'} + \mathrm{GSH} \rightarrow \mathrm{RSSG} + \mathrm{R'SH} \tag{3}$$

$$\mathrm{RSSG} + \mathrm{GSH} \rightarrow \mathrm{GSSG} + \mathrm{RSH} \tag{4}$$

Such reactions were generally monitored by coupling with glutathione reductase to the GSSG-dependent oxidation of NADPH.

$$\mathrm{GSSG} + \mathrm{NADPH} + \mathrm{H}^+ \rightarrow 2\ \mathrm{GSH} + \mathrm{NADP}^+ \tag{5}$$

It was assumed that both of the thiol-disulfide reactions (Eqns. 3 and 4) were catalyzed by thioltransferase. However, it could be argued that the first reaction (Eqn. 3), which does not involve a glutathione sulfenyl group in the scissile sulfur-sulfur bond, could be spontaneous and only the second reaction (Eqn. 4) be enzymatic. The studies of the thiolysis of cystamine presented in this paper (Fig. 1 and Table I) show clearly that glutathione or a glutathione derivative is not an obligatory reactant of the reactions catalyzed by thioltransferase. On the contrary, the enzyme shows a very broad specificity both towards thiols and disulfide substrates. The substrates include low-molecular-weight as well as protein thiols and disulfides. In an earlier study involving a mixed disulfide of glutathione and 3-carboxy-4-nitrobenzenethiol it was demonstrated that various *S*-nucleophiles were substrates for thioltransferase from rat liver [35]. However, these studies were carried out with partially purified enzyme and at an unphysiological pH value of 5.5. The present results therefore demonstrate more unambiguously the possible importance of alternative substrates in vivo.

It has previously been claimed that a major physiological role of thioltransferase is the reduction of low-molecular-weight disulfides and thiosulfate esters [1,7—11]. The results of the present study are in agreement with this statement and lend further support to the proposal that the enzyme catalyzes the scission of naturally occurring mixed disulfides of glutathione and protein sulfhydryl groups [11,12,14,15]. It has recently been shown that the enzymatic effect is not only an unspecific reaction with undefined cytoplasmic liver proteins, but also a specific effect on the reactivation of pyruvate kinase, which had been deactivated by reaction with GSSG [14].

Even if various thiols can serve as substrates for the above-mentioned reactions (Figs. 1 and 2, Tables I and II), it appears likely that for reduction of sulfur-sulfur bonds the physiological reductant is GSH, since GSH is the most abundant low-molecular-weight thiol in most cells [36]. The coupling to the subsequent reduction of GSSG catalyzed by glutathione reductase is also in line with this assumption. However, it should be stressed that all thiol-disulfide interchange reactions do not have to serve as a reduction of sulfur-sulfur bonds, but they can also be used to redistribute moieties of various symmetrical and unsymmetrical disulfides. Various mixed disulfides have been isolated from biological samples (see Ref. 7 for a review), and thioltransferase may have a role in the formation and recombination in addition to the reduction of such molecules. In some of these reactions other thiols than GSH may be important substrates.

The present study also shows that thioltransferase catalyzes the formation of mixed disulfides of proteins. Such reactions are, like their reversal, important in view of the diurnal variations of the level of protein-mixed disulfides in rat liver [18,28]. The reaction involving proteins and cystamine was investigated because of its suggested role in the formation of disulfides in protein biosynthesis [31]. The finding that relatively small amounts of mixed disulfides of proteins and cysteamine were formed may be interpreted as supporting the proposed role, if it is assumed that the oxidation of protein sulfhydryl groups takes place without accumulation of intermediary mixed disulfides. However, the clarification of this reaction was outside the scope of the present study and has to await further experiments.

The effect of thioltransferase on the scission of sulfur-sulfur bonds in disulfide-containing proteins, indicates that the enzyme may have a role in the intracellular degradation of proteins. The localization of protein degradation is commonly considered to be in the lysosomes, and a thioltransferase activity has been found in these organelles [37]. Even if the lysosomal thioltransferase has not yet been identified with the cytoplasmic enzyme, it should be noted that some proteins may also be degraded in the cytoplasm [38]. In agreement with earlier studies on the nonenzymatic thiolysis of protein disulfide bonds [32,33], it was found that denaturation was necessary to make disulfide bonds available for cleavage. The effect of the enzyme was very substantial when the velocity of the disulfide reduction was recalculated to the average concentration of thioltransferase in the cytosol. However, the presence of thioltransferase did not increase the fraction of accessible bonds in the protein substrate in comparison with that available for the nonenzymatic reaction. Therefore, the possible role of thioltransferase in protein catabolism is secondary to rate-limiting denaturation processes which bring about conformational changes and exposure of scissile bonds.

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