Thioltransferase Is a Specific Glutathionyl Mixed Disulfide Oxidoreductase†

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ABSTRACT: To study the substrate specificity and mechanism of thioltransferase (TTase) catalysis, we have used ^{14}C- and ^{35}S-radiolabeled mixed disulfides of cysteine and glutathione (GSH) with various cysteine-containing proteins. These protein mixed disulfide substrates were incubated with glutathione, glutathione disulfide (GSSG) reductase, and NADPH in the presence or absence of thioltransferase. Glutathione-dependent reduction of protein mixed disulfides was monitored both by release of trichloroacetic acid soluble radiolabel and by formation of GSSG in an NADPH-linked spectrophotometric assay. GSH-dependent dethiolation of [^{35}S]glutathione–papain mixed disulfide (papain–SSG) and the corresponding bovine serum albumin mixed disulfide (BSA–SSG) were catalyzed by thioltransferase (from human red blood cells) as shown by the radiolabel assay, and equivalent rates were measured by the spectrophotometric assay. Dethiolation of [^{35}S]hemoglobin–glutathione mixed disulfide (Hb–SSG) was also catalyzed by TTase. In contrast, TTase did not catalyze GSH-dependent dethiolation of [^{14}C]papain–SScysteine or [^{14}C]BSA–SScysteine as measured by the radiolabel assay. [^{14}C]Hb–SScysteine and Hb–SScysteamine also did not serve as substrates. In separate experiments, TTase from rat liver displayed analogous selectivity. Thus, thioltransferase (glutaredoxin) appears to be specific for glutathione-containing mixed disulfides. Apparent TTase catalysis of GSSG formation from the papain– and BSA–SScysteine mixed disulfides was observed by the spectrophotometric assay, but a lag phase occurred consistent with preenzymatic formation of GSScysteine which could serve as the actual TTase substrate. Two-substrate kinetic studies of TTase with GSH and GSScysteine gave patterns of parallel lines on double-reciprocal plots ($1/V$ vs $1/[S]$), consistent with a simple ping-pong mechanism involving a TTase–SSG intermediate.

Racker (1955) identified an intracellular enzyme activity in rat liver homogenate which catalyzed the reduction of disulfide bonds. Subsequent studies associated this activity with thioltransferase, a low molecular weight protein with a characteristic dithiol active site and a member of a family of ubiquitous thiol:disulfide oxidoreductase enzymes which includes thioredoxin and glutaredoxin (Holmgren, 1989; Mannervik et al., 1989). It has been suggested because of their close structural homology that thioltransferase and glutaredoxin may be alternative names for the same set of enzymes (Papayannopoulos et al., 1989). Thioltransferases (glutaredoxins) have been distinguished from the thioredoxins by their ability to be reduced by glutathione. Moreover, oxidized thioredoxins are recycled by coupling to a specific flavoprotein, thioredoxin reductase, that does not reduce thioltransferases. Thioltransferase enzymes are recycled by coupling to GSH[1] and GSSG reductase that does not recycle thioredoxins. In fact, standard spectrophotometric assays of TTase rely on coupling the formation of GSSG to the utilization of NADPH by GSSG reductase. As long as excess GSSG reductase is added, the coupled assay effectively measures the overall rate of formation of the final product GSSG in 1:1 correspondence to the observed NADPH oxidation. Rates of the intermediate steps in the reaction sequence, however, are undetected. By use of the coupled assay, TTase catalysis has been implicated in the reduction of a wide variety of low molecular weight disulfides and protein disulfides, and it is thought to be important in the maintenance of sensitive intracellular thiols (Axelsson et al., 1978; Shen et al., 1991; Mieyal et al., 1991a,b).

Early studies suggested that TTase could catalyze both steps 1 and 2 in the sequential formation of GSSG:

$$RSSR' + GSH \rightleftharpoons RSH + GSSR' \quad (1)$$

$$GSSR' + GSH \rightleftharpoons GSSG + R'SH \quad (2)$$

$$GSSG + NADPH \overset{GRase}{\rightleftharpoons} 2GSH + NADP \quad (3)$$

This has led to the use of hydroxyethyl disulfide, cysteine thiosulfate, and cystine as model substrates in the coupled assay system (Axelsson et al., 1978; Holmgren, 1985). Using rat liver TTase, Axelsson and Mannervik (1980) suggested that TTase was capable of catalyzing reduction of both glutathione-containing as well as non-glutathione-containing disulfides. Although catalysis of the first step of reduction of non-glutathione-containing disulfides cannot be observed directly by the standard spectrophotometric assay, there is ample evidence that TTase does catalyze, and has shown an

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[1] Abbreviations: BSA, bovine serum albumin; cpm, counts per minute; cyanomet-Hb, cyanomethemoglobin; DMSO, dimethyl sulfoxide; DNP, dinitrophenylsulfenyl chloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GSScysteine, glutathione–cysteine mixed disulfide; GSSG, glutathione disulfide; GSSG reductase, glutathione disulfide reductase; Hb, hemoglobin; HEDS, hydroxyethyl disulfide; HPLC, high-pressure liquid chromatography; HRBC, human red blood cell(s); met-Hb, ferric hemoglobin; IEF, isoelectric focusing; MP, mercaptopyridine; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NTSB, 2-nitro-5-thiosulfobenzoate; oxyHb, oxyhemoglobin; PDS, 2,2′-pyridine disulfide; –SScysteine, cysteine mixed disulfide; –SSG, glutathione mixed disulfide; TCA, trichloroacetic acid; TTase, thioltransferase.

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apparent K_M preference for, the reduction of glutathione-containing disulfides (eq 2) (Axelsson et al., 1978; Mieyal et al., 1991b).

A general mechanism has been proposed for catalysis of disulfide reduction by thioltransferase whereby the intramolecular disulfide form of the enzyme is an intermediate in the overall reaction after formation of the reduced products of the disulfide substrate; the enzyme disulfide is then reduced to complete the cycle (Gan & Wells, 1987). This mechanism would be expected to generate a ping-pong kinetic pattern when both substrates are varied. Instead we observed a sequential kinetic pattern, using the coupled assay with GSH and the non-glutathione-containing prototype disulfide substrate HEDS, suggesting that GSH played a more specific role in the reaction scheme than simply reducing the enzyme disulfide (Mieyal et al., 1991b).

To dissect the mechanism of TTase catalysis further and observe the rate of the first step of the reaction (eq 1), we developed radiolabel and HPLC dethiolation assays in which GS-containing and non-GS-containing mixed disulfides of proteins were used to measure the rates of dethiolation by GSH in the presence or absence of TTase. In so doing we discovered that TTase effectively catalyzes GSH-dependent dethiolation of GS-containing mixed disulfides (eq 2) but is essentially ineffective in catalyzing the dethiolation of non-glutathione-containing disulfides (eq 1). Thus, TTase appears to be highly selective for glutathionyl mixed disulfide substrates. Furthermore, two-substrate kinetic studies of TTase-catalyzed dethiolation of glutathione-containing mixed disulfides gave patterns consistent with a simple ping-pong mechanism involving a TTase–SSG intermediate.

EXPERIMENTAL PROCEDURES

Materials. Sephadex G-25 and G-75, DEAE-Sephacyl and DEAE-cellulose, agarose IEF, and ampholytes 6–8 were obtained from Pharmacia LKB. 2,4-Dinitrophenylsulfenyl chloride was purchased from Aldrich. Acrylamide, bisacrylamide, tricine, sodium dodecyl sulfate, and unstained low molecular weight standards were obtained from Bio-Rad. NADPH was obtained from Boehringer Mannheim. [^{35}S]Glutathione (50–100 Ci/mmol) was obtained from NEN. [^{14}C]Cystine (50 Ci/mmol) and Bio-Safe II counting cocktail were obtained from Research Products International Corp. Hydroxyethyl disulfide, glutathione and glutathione disulfide, dithiothreitol, ethylenediaminetetraacetic acid, 2,2′-pyridine disulfide, cystine, glutathione reductase (type IV from bakers' yeast or type VI from spinach), iodoacetamide, β-mercaptoethanol, Latex papain, Tris base, and 5,5′-dithiobis(2-nitrobenzoic acid) were supplied by Sigma. Bovine serum albumin (RIA Grade) was from U.S. Biochemicals. *Escherichia coli* thioredoxin was obtained from Calbiochem, and *E. coli* thioredoxin reductase was from American Diagnostica Inc. Trichloroacetic acid, dimethyl sulfoxide, and mono- and dibasic potassium phosphate were purchased from Fisher. All other reagent-grade chemicals and solvents were obtained from standard sources. Thioltransferase was purified to apparent homogeneity from human red blood cells as described previously (Mieyal et al., 1991a). Partially purified TTase gave results identical to those with pure TTase. Partially purified rat liver TTase was prepared as described previously (Mieyal et al., 1991b).

Assays for Sulfhydryls and Disulfides. Sulfhydryl content of proteins was determined with DTNB by the method of Ellman (1959); $\epsilon_{412nm} = 13.6\ mM^{-1}\ cm^{-1}$. Because hemoglobin absorbs strongly at 412 nm, PDS was used to titrate Hb sulfhydryls by difference spectroscopy (ϵ_{341nm} of mercaptopyridine = $8.08\ mM^{-1}\ cm^{-1}$; Stuchbury et al., 1975).

Disulfide content of proteins was determined relative to a standard curve generated with GSSG, according to the method of Thannhauser (1984) using NTSB. Because the NTSB method determines the sum of disulfides and sulfhydryls present, the number of preexisting sulfhydryls was determined independently by the DTNB assay and subtracted from the total number found in the NTSB assay.

Protein Determinations. Protein concentrations were determined by the Pierce micro-BCA method in microtiter plates (volume = 0.2 mL) with BSA as a standard, according to the manufacturer's protocol. BSA and papain concentrations were determined spectrophotometrically by use of their respective extinction coefficients, $\epsilon_{280nm} = 44$ and $56\ mM^{-1}\ cm^{-1}$ (Sober & Harte, 1973; Brocklehurst et al., 1973). Hemoglobin concentrations were determined as cyanoferric hemoglobin ($\epsilon_{541nm} = 44\ mM^{-1}\ cm^{-1}$; Van Kampen & Zijlstra, 1961).

Standard Coupled Assay for Thioltransferase. Spectrophotometric determinations of TTase activity were carried out according to Mieyal et al. (1991a). The slope of the linear portion of the time course of decreasing A_{340nm} for the minus TTase control was subtracted from the slopes of the TTase-containing samples in order to determine the TTase-dependent rates. One unit of TTase activity is defined as 1 μmol of NADPH oxidized per GSSG formed per min (ϵ_{340nm} for NADPH = $6.2\ mM^{-1}\ cm^{-1}$).

Spectrophotometric Assay for Thioredoxin Activity. The thioredoxin assay at 30 °C contained 0.1 M potassium phosphate, pH 7.5, 0.2 mM NADPH, 0.1 μM *E. coli* thioredoxin reductase, and 0.15 mM mixed disulfide substrate, in the absence or presence of 0.5–5 μM *E. coli* thioredoxin. Reactions were initiated by addition of the substrate, and the time course of decreasing A_{340nm} (NADPH oxidation) was monitored. No reaction occurred in the absence of thioredoxin or thioredoxin reductase.

Radiolabel Dethiolation Assay. Dethiolation of ^{35}S- or ^{14}C-labeled protein mixed disulfides was carried out by a procedure analogous to that of Mannervik and Axelsson (1980). Radiolabeled samples were incubated at 30 °C with 0.1 M potassium phosphate, pH 7.5, 2 units/mL yeast GSSG reductase, 0.2 mM NADPH, and 0.5 mM GSH, in the absence or presence of TTase (see legends to figures and tables). No reaction occurred in the absence of GSH. The thioredoxin-catalyzed dethiolation assay at 30 °C contained 0.1 M potassium phosphate, pH 7.5, 0.2 mM NADPH, and 0.1 μM *E. coli* thioredoxin reductase, in the absence or presence of 0.5–5 μM *E. coli* thioredoxin. No reaction occurred in the absence of thioredoxin or thioredoxin reductase. Reactions with papain mixed disulfides were performed in 0.1 M Tris-HCl buffer, pH 7.5, to limit the proteolytic activity of the mercaptopapain (Kimmel & Smith, 1953). After preincubation of the mixtures of 30 °C for 5 min, reactions were started by the addition of radiolabeled disulfides. Spontaneous rates of dethiolation were determined in the absence of TTase. Reactions were terminated at various times by addition of one volume of ice-cold 20% TCA followed by centrifugation for 1 min at top speed in a microcentrifuge. An aliquot of the supernate was removed and added to 5 mL of Bio-Safe II scintillation cocktail and counted in a Beckman LS 5000CE liquid scintillation counter. Net release of radioactivity in the supernate was determined by subtracting background radioactivity. Background cpm were measured in control samples containing the protein mixed disulfides in the absence of reducing agents; alternatively, the linear region of time

courses of radioactivity release or the linear region of concentration dependences were extrapolated to the origin. Initial specific radioactivities of all radiolabeled mixed disulfides were within the range 0.3–1.5 Ci/mol. To correct for radioactive decay of ^{35}S compounds, aliquots of the appropriate stock solutions were saved and used to determine the specific radioactivity on the days of use. The lowest specific radioactivity used for any experiment was >0.2 Ci/mol. Concentration dependence of mixed disulfide substrates was determined under conditions of linear time and enzyme concentration dependence determined in separate experiments. Apparent V_{max} and K_M values were estimated by nonlinear least-squares fitting to the Michaelis–Menten equation (Caceci & Cacheris, 1984).

Two-Substrate Kinetics. GSScysteine and GSH or [^{35}S]MetHb–SSG and GSH were varied independently to produce a 4 × 4 matrix in each case. Rates of enzymatic dethiolation were determined within the linear range of time and enzyme concentration. Enzyme sources were pure HRBC TTase, partially purified HRBC TTase, and partially purified rat liver TTase. The V vs [S] data for each fixed concentration of the second substrate were fit to a hyperbola by nonlinear regression analysis in order to obtain individual estimates of apparent V_{max} and K_M. The primary data were then transformed into double-reciprocal plots for ease of pattern recognition.

Preparation of Glutathione–Cysteine Mixed Disulfide. GSScysteine was prepared by the method of Eriksson and Eriksson (1967). Purity of GSScysteine was verified by quantitative amino acid analysis. GSScysteine at 1 mM was reduced by 5 mM DTT in 10 mM potassium phosphate, pH 7.5, for 10 min at 25 °C and the thiol groups were reacted with 50 mM iodoacetamide. Amino acid composition of the reduced, iodoacetamide-treated GSScysteine was compared to that of (carboxamidomethyl)glutathione on a Beckman 119CL amino acid analyzer with the standard three-buffer system (Haas & Rosenberry, 1985). Amino acid analysis showed the expected ratio for (carboxyamidomethyl)cysteine: glycine:glutamic acid of 2:1:1.

Preparation of 2,4-Dinitrophenyl–Glutathionyl and –Cysteinyl Disulfides. DNP–SSG was prepared by the method of Faulstich et al. (1984). GSH (0.5 mmol) and 50 μCi of [^{35}S]GSH were reacted with 0.55 mmol of 2,4-dinitrophenylsulfenyl chloride in 1 mL of 98% formic acid at 25 °C. The melting point of DNP–SSG was 187 °C as reported (ϵ_{408nm} for DNP = 12 700 M^{-1} cm^{-1}; Faulstich et al., 1984). DNP–SScysteine was prepared by the procedure of Drewes and Faulstich (1990). Cysteine (5.7 μmol) was incubated for 2 h with NH_4HCO_3 (to pH 8.0) and 22 μCi of [^{14}C]cystine (97 μCi/μmol) in 0.2 mL of H_2O under N_2 and then dried in vacuo. The resulting [^{14}C]cysteine was dissolved in 0.4 mL of formic acid and reacted with 10 μmol of 2,4-dinitrophenylsulfenyl chloride. The melting point for [^{14}C]DNP–SScysteine was 180–185 °C.

Preparation of Bovine Serum Albumin–[^{14}C]Cysteine and –[^{35}S]Glutathione Mixed Disulfides. Mercapto-BSA was prepared by a modification of the procedure of Hartley et al. (1962). All steps were at 4 °C. BSA (10 g) in 100 mL of 1 mM potassium phosphate, pH 7.0, was added to DEAE-Sephacel (5.5 cm × 28 cm) equilibrated in the same buffer. The column was washed for 3 h with buffered mercaptoethanol (10 mM; 200 mL) and then with 300 mL of buffer. Then proteins were eluted with a linear gradient formed with 1 L each of 0.1 M and 0.2 M potassium phosphate, pH 7.0. Mercapto BSA eluted first. Sulfhydryl to BSA ratios were 0.9–1 and were stable for at least 12 months when stored at concentrations ≥ 1 mM at –20 °C. SDS–Tricine–PAGE analysis showed mercapto-BSA to be a monomer of 66 kDa and 95% pure.

BSA–SScysteine was prepared from mercapto-BSA and [^{14}C]cystine according to the method of Isles et al. (1962). Radiolabel incorporation, sulfhydryl content, and disulfide content showed 0.8–0.9 cysteine/BSA. Yields of BSA–SScysteine were 80–95%.

[^{35}S]BSA–SSG was prepared by combining mercapto-BSA (1 mM) in 0.1 M potassium phosphate, pH 7.5, with 1 mM [^{35}S]GSH. Diamide (1 mM) was added and the solution was incubated at room temperature for 30 min, and then diamide and GSSG were removed by gel filtration. The BSA-containing fractions were pooled, concentrated via ultrafiltration, and reincubated with [^{35}S]GSH and diamide as above. Radiolabel incorporation, sulfhydryl content, and disulfide content showed 0.9–0.95 glutathione/BSA. Yields of BSA–SSG were >90%.

Preparation of Papain–[^{14}C]Cysteine and –[^{35}S]Glutathione Mixed Disulfides. Papain (24 kDa) as received in 0.05 M sodium acetate, pH 4.5, was converted into mercaptopapain according to Brocklehurst and Little (1973). The papain was diluted to 0.22 mM with 10 mM DTT in water, the pH was adjusted to 8.0 with 1 N NaOH, and the solution was incubated for 1 h at 30 °C. DTT was removed by gel filtration. SDS–Tricine–PAGE analysis indicated a purity of >90%. Sulfhydryl contents for multiple preparations gave sulfhydryl/papain ratios of 0.9–1.2. Yields ranged from 70 to 100%.

[^{35}S]DNP–SSG or [^{14}C]DNP–SScysteine (0.2 mM) in 1% ammonium carbonate buffer, pH 8.5, was reacted with mercaptopapain (0.1 mM) in 10 mM KCl and 1 mM EDTA for 15 min at room temperature. Incorporation of [^{35}S]GSH (or [^{14}C]cysteine) into papain was monitored by observing A_{408nm} and by measuring radioactivity, sulfhydryl content, and disulfide content. Reactants were removed by gel filtration. The stoichiometry of incorporation was 0.7–1. The yield of papain protein was usually >90%.

Preparation of Hemoglobin–[^{14}C]Cysteine and –[^{35}S]Glutathione Mixed Disulfides. Human oxyHb was purified as described by Ferraiolo et al. (1984) and converted to Hb–SScysteine by incubating 0.125 mM oxyHb at 4 °C with 0.625 mM [^{14}C]cystine (0.6 Ci/mol; specific radioactivity of Cys equivalents = 0.3 Ci/mol). Progress of the reaction was followed to completion (several days) by removing aliquots periodically and measuring incorporation of radiolabel into TCA-precipitated protein. Excess cystine was removed by Sephadex G-75 chromatography, and protein was concentrated by an ultrafiltration cell (Amicon YM-5). Analysis of purity of Hb–SScysteine (>95%) was carried out as described below for Hb–SSG. Yield was 95%.

Human oxyHb was purified as described above and converted to Hb–SSG by the procedure of Garel et al. (1986), with [^{35}S]GSH (specific activity 0.5 Ci/mol). MetHb–SSG was formed from oxyHb–SSG by addition of $K_3Fe(CN)_6$ in a 1.25-fold molar excess over heme, followed by gel filtration. Incorporation of radiolabel ranged from 1.8 to 2.0 GSH/Hb (i.e., 0.9–1.0 GSH/β-subunit). Spectrophotometric analysis gave the expected visible spectra for oxy- and metHb derivatives. Isoelectric focusing, as described previously (Mieyal et al., 1991a), showed that Hb substrates were >90% pure. G-75 Sephadex column chromatography showed Hb–SSG to have a molecular mass of 64 kDa, consistent with maintenance of the tetrameric structure. Yields ranged from

50 to 70%. Unlabeled Hb–SSG was prepared as was [^{35}S]Hb-SSG, and its purity was verified by IEF.

Preparation and Assay of Cyanomethemoglobin–Cysteamine Mixed Disulfide. Purified oxyHb was incubated with a 10-fold excess of cystamine for 1 h at room temperature, and then excess cystamine was removed by gel filtration. The oxyHb–SScysteamine was converted to cyanometHb–SS-cysteamine by the addition of a 1.25-fold excess over heme of KCN and $K_3Fe(CN)_6$, followed by gel filtration. Analytical IEF band shift and sulfhydryl content showed cyanometHb–SScysteamine to be about 95% pure. Aliquots of a 0.33 mM stock solution of cyanometHb–SScysteamine in 10 mM potassium phosphate, pH 7.5, were stored frozen at −70 °C.

CyanometHb–SScysteamine (0.15 mM) was incubated with 2.0 mM GSH, 0.2 mM NADPH, 2 units/mL yeast GSSG reductase, and 0.1 M potassium phosphate, pH 7.5, in the presence or absence of 0.6–1.6 units/mL TTase at 30 °C. At various times (including zero time) samples were diluted 1:6 in deionized water and 20 μL was injected into the HPLC. Dethiolation of cyanometHb–SScysteamine was determined by use of a Dionex Propac PA1 (4 × 250 mm) anion-exchange HPLC column with a linear gradient of NaCl (0–0.2 M) in 50 mM Tris-HCl, pH 7.5, formed over 1 h. CyanometHb–SScysteamine eluted at 24.3 ± 0.72 min and cyanometHb–SH eluted at 26.0 ± 0.59 min (n = 7; ±SE) as detected by A_{415nm}, and they were baseline resolved. The areas of the respective peaks for the different time point samples were measured by automatic integration and used to quantitate the rate of dethiolation. The change in the Hb–SH peak area at each time was calculated by subtracting the corresponding area at zero time. In order to determine whether Hb–SSG might be a product of the reaction, it was confirmed in a separate test that the peak for Hb–SSG was well resolved from those of Hb–SScysteamine and Hb–SH.

RESULTS

The equations below depict possible reactions of GSH with protein mixed disulfides (Gilbert, 1990). Equations 4a,b show the potential radiolabel distributions when the disulfide is labeled on the nonprotein moiety. Equations 5a,b show the unlabeled disulfide reacting with radiolabeled GSH. The data in this article indicate that the GSH thiol reacts preferentially with the nonprotein sulfur (eqs 4a and 5a) and that thioltransferase (glutaredoxin) effectively catalyzes the reaction only when R is a glutathionyl moiety.

$$\text{protein–SSR}^* + \text{GSH} \rightleftharpoons \begin{cases} \text{protein–SH} + \text{GSSR}^* & (4a) \\ \text{or} \\ \text{protein–SSG} + {}^*\text{RSH} & (4b) \end{cases}$$

$$\text{protein–SSR} + {}^*\text{GSH} \rightleftharpoons \begin{cases} \text{protein–SH} + {}^*\text{GSSR} & (5a) \\ \text{or} \\ \text{protein–SSG}^* + \text{RSH} & (5b) \end{cases}$$

Reduction of Hemoglobin Mixed Disulfides. Native hemoglobin has one sulfhydryl group on each β-subunit that can form mixed disulfides with other thiol compounds (see Experimental Procedures). Thus, it is a well-defined and convenient model protein substrate for studying catalysis of disulfide reduction, and we had previous evidence that TTase catalyzed dethiolation of Hb–SSG (Mieyal et al., 1991a). Comparison of the rates of GSH-dependent reduction of [^{35}S]Hb–SSG and [^{14}C]Hb–SScysteine in the presence and absence of the enzyme showed that TTase did catalyze dethiolation of the *glutathionyl* substrate (Figure 1A), whereas

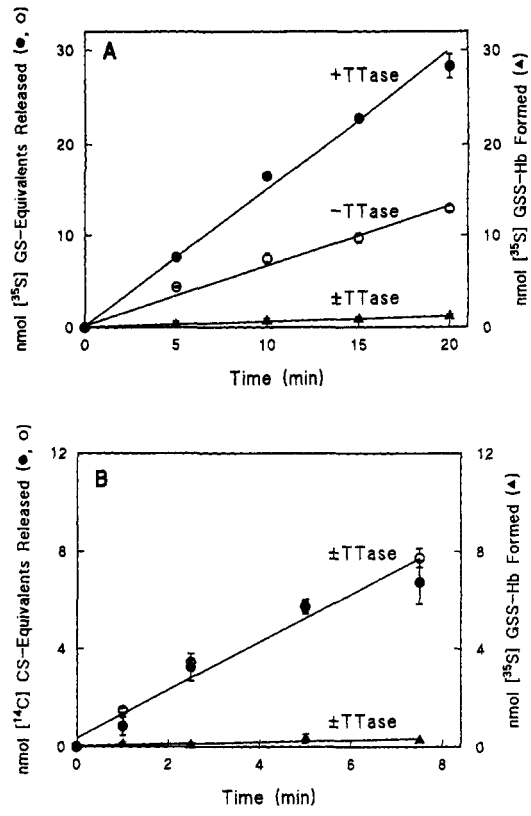

FIGURE 1: Time dependence of dethiolation of [^{35}S]oxyHb–SSglutathione and [^{14}C]oxyHb–SScysteine by GSH ± TTase. (A) Time dependence of dethiolation of [^{35}S]oxyHb–SSG by GSH ± TTase. Reaction mixtures (1 mL) at 30 °C contained 0.1 M potassium phosphate, pH 7.5, 0.15 mM [^{35}S]oxyHb–SSG, 0.5 mM GSH, 0.2 mM NADPH, and 2 units/mL yeast GSSG reductase, in the absence of presence of 0.64 unit/mL HRBC TTase. Dethiolation was measured by the radiolabel assay (see Experimental Procedures). Incorporation of [^{35}S]GSH into Hb was measured as described above except that *unlabeled* oxyHb–SSG was used, and the TCA-precipitated pellet (three times washed and dissolved in 1 N NaOH) was assayed rather than the supernate. Open circles correspond to samples without TTase; closed circles represent TTase-containing samples. Triangles represent the incorporation of [^{35}S]GSH into Hb. The minimum detectable radiolabel incorporation would correspond to <0.1% of the theoretical maximum incorporation. Data have been normalized to the amount of product formed per 1 mL of reaction mixture. Each data point represents the mean of three separate experiments ± SE. Where error bars are not easily apparent, they occur within the symbols. (B) Time dependence of dethiolation of [^{14}C]oxyHb–SScysteine by GSH ± TTase. Experiments were carried out as described for panel A, except that [^{14}C]oxyHb–SScysteine replaced [^{35}S]oxyHb–SSG.

TTase (up to 0.6 unit/mL) did *not* catalyze dethiolation of the *cysteinyl* mixed disulfide (Figure 1B). Because release of radioactivity from a radiolabeled protein mixed disulfide (protein–SSR*) may arise from nucleophilic attack of GSH on either sulfur atom of the disulfide bond (eqs 4a,b), the two possibilities were distinguished by measuring [^{35}S]GSH incorporation into unlabeled protein mixed disulfides (eqs 5a,b). Little incorporation of [^{35}S]GSH into precipitated Hb was observed under the same conditions where substantial release of radioactivity from the labeled mixed disulfides of Hb was measured (Figure 1, closed triangles). Thus the reactions of the mixed disulfides of Hb with GSH are depicted by eqs 4a and 5a.

Hb–SScysteamine, another non-glutathione mixed disulfide, was also tested. HPLC was used to monitor the reaction, because radiolabeled cysteamine was not commercially available. GSH-dependent dethiolation occurred, but no catalysis by TTase was observed (Table I), even in the presence of

Table I: HPLC Measurement of Hemoglobin–Cysteamine Dethiolation[a]

time (min)	Hb-SH (nmol mL^{-1})[b] –TTase	+TTase
30	18.5 ± 1.0	19.5 ± 3.0[c] 17.3 ± 0.8[d]
120	52.5 ± 2.3	52.5 ± 2.3

[a] Reaction mixtures contained 0.1 M potassium phosphate, pH 7.5, 2.0 mM GSH, 0.2 mM NADPH, and 2 units/mL GSSG reductase, in the absence or presence of 0.6–1.6 units/mL TTase at 30 °C. The reactions were initiated by the addition of Hb–SScysteamine to 0.15 mM. At various times, products were analyzed by anion-exchange HPLC (see Experimental Procedures). [b] Values of nanomoles of Hb-SH formed per milliliter at each time (relative to a zero time; see Experimental Procedures) were calculated by multiplying fractional peak area [ΔHb–SH peak area/(sum of Hb–SScysteamine + Δ Hb–SH peak areas)] by the concentration of Hb–SScysteamine in the original reaction mixture. Values are the mean of three separate experiments ± SE. [c] HRBC TTase. [d] Rat liver TTase.

relatively large amounts of the enzyme (0.64–1.6 units/mL). Analogous results were obtained in the presence of rat liver TTase (Table I). Spontaneous dethiolation of Hb–SScysteamine by GSH was observed by appearance of the Hb–SH peak, and no peak corresponding to Hb–SSG appeared. Thus, the only detectable reaction with GSH was conversion of Hb–SScysteamine to Hb–SH, again showing the preference depicted by eqs 4a and 5a.

Reduction of Papain and BSA Mixed Disulfides. To determine whether discrimination by TTase between glutathione- and non-glutathione-containing disulfides extended to substrates other than hemoglobin mixed disulfides, alternative protein and small molecule disulfides were studied. Like hemoglobin, papain and BSA contain single cysteinyl thiol groups that can form mixed disulfides. Hence, the cysteinyl and glutathionyl mixed disulfides of BSA and papain were made and tested as substrates for TTase.

Rates of [^{35}S]papain–SSG reduction by GSH were measured both by release of radioactivity and by the GSSG reductase-coupled spectrophotometric assay (see Experimental Procedures). TTase catalyzed the GSH dependent reduction of papain–SSG, and the spectrophotometric and radiolabel assays gave essentially the same results, as expected (Table II). Because the spectrophotometric assay measures formation of GSSG specifically, the radioactivity released from [^{35}S]papain–SSG was [^{35}S]GSSG, consistent with selective attack of GSH on the glutathionyl moiety of [^{35}S]papain–SSG (eq 4a).

Reduction of papain–SScysteine was studied also by both assays. Time-dependent formation of GSSG from papain–SScysteine measured by the spectrophotometric assay showed an increased rate in the presence of TTase (Table II). In contrast, TTase did not catalyze release of radioactivity from [^{14}C]papain–SScysteine even when relatively large amounts of the enzyme (0.008–1.1 units/mL) were added (Table II). Analogous results were obtained with partially purified rat liver TTase (data not shown).

Likewise, apparent catalysis of BSA–SScysteine reduction by TTase was observed by the GSSG reductase-coupled spectrophotometric assay (Mieyal et al., 1991b; Table II), but no catalysis by TTase was observed by the radiolabel assay of [^{14}C]BSA–SScysteine dethiolation (Table II). Increasing the TTase concentration (40-fold) and the substrate concentration (3-fold) still failed to show any catalysis of the dethiolation reaction. With [^{35}S]BSA–SSG, however, GSH-dependent release of radioactivity was catalyzed by TTase, and similar rates were observed with the spectrophotometric assay (Table II).

Table II: Rates of Dethiolation for Various Protein Mixed Disulfides ± Thioltransferase[a]

substrate	radiolabel assay (nmol min^{-1} mL^{-1})	TTase (units/mL)	spectrometric assay (nmol min^{-1} mL^{-1})	TTase (units/mL)
papain–SSG	7.2		7.7	
	20.7	0.008	20.0	0.008
papain–SSCys	65.3		3.0	
	65.6	0.008–1.1	12.0	0.008
BSA–SSG	3.5		2.4	
	6.2	0.016	5.7	0.016
BSA–SSCys	27.2		6.6	
	27.6	0.01–0.64	13.2	0.008
oxyHb–SSG	0.64		N/A[b]	N/A
	1.4	0.64	N/A	N/A
oxyHb–SSCys	1.0		N/A	N/A
	0.94	0.64	N/A	N/A

[a] Rate values represent the slopes of regression analyses of the linear phases of product formation vs time graphs, except for papain–SSCys, where the values correspond to the extent of radioactivity released in 1 min. For each experiment at least three determinations were made at each time point; the SE values in all cases were <10% of the mean. All regression values were ≥0.98. Reactions were performed at 30 °C in 0.5 mM GSH, 0.2 mM NADPH, 2 units/mL GSSG reductase, and 0.1 M potassium phosphate, pH 7.5 (except papain mixed disulfides were run in 0.1 M Tris-HCl, pH 7.5; see Experimental Procedures). All protein mixed disulfides were at 0.15 mM. Spectrophotometric assay measures the loss of NADPH at A_{340nm} coupled to GSSG formation, and the radiolabel assay measures TCA-nonprecipitable counts (see Experimental Procedures). [b] N/A, not applicable; spectrophotometric assays were not performed with the hemoglobin substrates, because the heme absorbance interferes with accurate determination of A_{340} changes.

Basis for Apparent TTase Catalysis of Protein–SSCysteine Reduction Only When Measured as GSSG Formation. A lag phase during the spectrophotometric assay of a variety of non-glutathionyl disulfide substrates has been observed previously (Axelsson et al., 1978; Mieyal et al., 1991b). Figure 2 shows a comparison of the initial time courses for GSH-dependent GSSG formation from BSA–SScysteine and BSA–SSG measured spectrophotometrically. At equal concentrations of mixed disulfide, only BSA–SScysteine showed a lag phase. Analogous results were found for other pairs of substrates, e.g., CoASSCoA vs CoASSG (i.e., the non-GS-containing disulfide *only* displayed a lag phase; data not shown). These results suggest that the GS-moiety is incorporated into a mixed disulfide substrate before TTase catalysis occurs. Thus, with the protein–SScysteine compounds, GSScysteine is formed spontaneously and would be the actual substrate for TTase catalysis as measured by the spectrophotometric assay (eqs 6 and 7):

$$\text{protein–SSCys} + \text{GSH} \rightleftharpoons \text{protein–SH} + \text{GSSCys} \quad (6)$$

$$\text{GSSCys} + \text{GSH} \overset{\text{TTase}}{\rightleftharpoons} \text{Cys-SH} + \text{GSSG} \quad (7)$$

Accordingly, GSScysteine was synthesized and found to be a superior substrate for TTase with a low apparent K_M and a high turnover number (Table III).

Also shown in Table III is a summary of kinetic data for reduction of various mixed disulfides. Substrate concentration dependence of dethiolation of all GS-containing mixed disulfide substrates in the presence of 0.5 mM GSH followed typical Michaelis–Menten kinetics with HRBC TTase and showed an 80-fold range of apparent turnover numbers (27–2200 min^{-1}). Apparent K_M values for protein mixed disulfide substrates showed a 3-fold range, while the K_M for GSScysteine was 9-fold lower than that for any other disulfide tested. Apparent rate constants for the nonenzymatic spontaneous reactions with GSH varied over a 200-fold range (Table III).

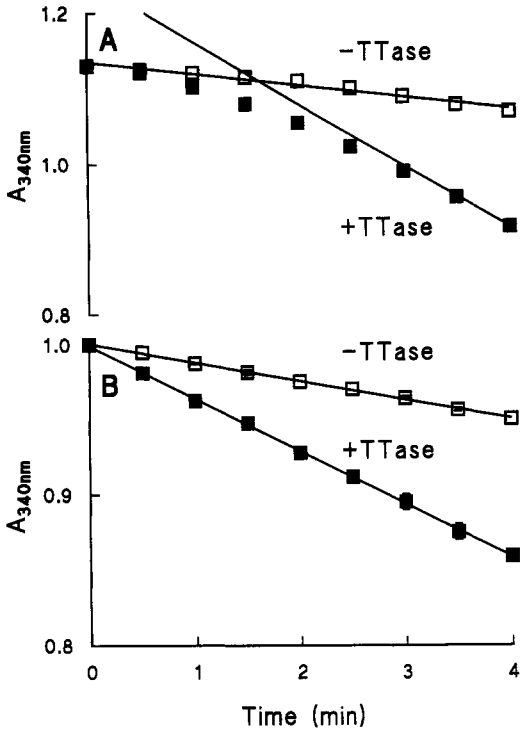

FIGURE 2: Initial time courses of GSH-dependent reduction of BSA–SScysteine and BSA–SSglutathione measured by the spectrophotometric coupled assay. (A) Initial time course for TTase-catalyzed GSSG formation from BSA–SScysteine and GSH. Reaction mixtures (1 mL) at 30 °C contained 0.1 M potassium phosphate, pH 7.5, 0.15 mM BSA–SScysteine, 0.5 mM GSH, 0.2 mM NADPH, 2 units/mL yeast GSSG reductase, and 0.016 unit/mL HRBC TTase. Reactions were initiated by addition of BSA–SScysteine with a mixing device and followed spectrophotometrically as described under Experimental Procedures. The upper line (open squares) corresponds to the time course in the absence of TTase, and the lower line (closed squares) represents extrapolation of the later linear portion of the time course in the presence of TTase (not shown completely). Data have been normalized to the amount of product formed per 1 mL of reaction mixture. Each data point represents the mean of three separate experiments ± SE. Where error bars are not easily apparent, they occur within the symbols. (B) Initial time courses for GSSG formation from BSA–SSG and GSH ± TTase. The upper line (open squares) corresponds to the time course in the absence of TTase, and the lower line (closes squares) corresponds to the time course in the presence of TTase. Experiments were performed as described for panel A, except that BSA–SSG replaced BSA–SScysteine.

Catalysis of Reduction of Protein Mixed Disulfides by E. coli Thioredoxin. The data above documented the selectivity of TTase for GS-containing disulfides. To test whether this property was peculiar to TTase, the *E. coli* thioredoxin system was tested in an analogous fashion. Thioredoxin catalyzed release of radioactivity from [^{14}C]papain–SScysteine and [^{35}S]BSA–SScysteine and the observed rates were in good agreement with those observed by spectrophotometric analysis (Table IV). Thioredoxin also catalyzed dethiolation of [^{35}S]metHb–SScysteine and [^{35}S]metHb–SSG mixed disulfides (Table IV). Thus, thioredoxin apparently does not discriminate between GS-containing and non-GS-containing mixed disulfide substrates as does TTase.

Two-Substrate Kinetics for GSH and GSSCysteine. Figure 3 shows the pattern of double-reciprocal plots for variation of GSScysteine concentration at several fixed concentrations of GSH as measured by the spectrophotometric assay. A similar pattern was observed when GSH was the variable substrate (not shown). Analogous parallel line patterns were observed when two-substrate kinetic experiments were performed with [^{35}S]metHb–SSG and GSH as measured by the radiolabel assay with HRBC TTase (data not shown). The parallel line pattern observed (Figure 3) is consistent with a ping-pong mechanism (see Discussion). In separate experiments, rat liver TTase also gave a ping-pong kinetic pattern when tested with GSScysteine and GSH (data not shown).

Table III: Kinetic Parameters for Catalyzed and Spontaneous Reactions of GSH with Disulfides[a]

substrate	$K_{M(app)}$[b] (mM)	$V_{max(app)}$[c] (min^{-1})	V_{max}/K_M (M^{-1} min^{-1})	$k_{(app)}$[d] (M^{-1} min^{-1})
Spectrophotometric Assay				
GSSCys	0.035	2200	63×10^6	1200
BSA–SSCys[e]	0.22	1080		
Radiolabel Assay				
BSA–SSG	0.44	1800	4.1×10^6	26
metHb–SSG	0.33	133	0.4×10^6	36
oxyHb–SSG	0.63	27	0.04×10^6	19
BSA–SSCys	N/A[f]	N/A		160
papain–SSCys	N/A	N/A		3700
oxyHb–SSCys	N/A	N/A		17

[a] Reaction mixtures at 30 °C contained 0.1 M potassium phosphate, pH 7.5, 0.5 mM GSH, 0.2 mM NADPH, and 2 units/mL GSSG reductase, in the presence or absence of TTase. Spectrophotometric assay measures the loss of NADPH at A_{340nm} coupled to GSSG formation, and the radiolabel assay measures TCA-soluble counts (see Experimental Procedures). [b] $K_{M(app)}$ was determined by nonlinear fit of velocity vs [substrate] curves. Each value of K_M and V_{max} represents the analysis of three separate experiments with at least five concentrations of the disulfide substrate, with [GSH] maintained at 0.5 mM. [c] Apparent turnover numbers at 0.5 mM GSH were calculated by multiplying the V_{max} (millimoles of product per minute per unit of TTase) by the specific activity (110 units/mg) and by the molecular weight (11 300 mg/mmol) of pure HRBC TTase. [d] Reaction mixtures for determination of nonenzymatic second-order rate constants contained 0.1 M potassium phosphate, pH 7.5 (except papain–SScysteine; 0.1 M Tris-HCl, pH 7.5) at 30 °C, with 0.15 mM GSH and 0.15 mM oxidized substrate (and 2 units/mL GSSG reductase for spectrophotometric measurements). Second-order rate constant (k_{app}) was determined from the slope of a plot of 1/[oxidized substrate] remaining vs time. [e] Mieyal et al. (1991b). [f] N/A, not applicable; i.e., no TTase catalysis was observed. A maximum estimate of the potential catalytic activity of TTase for RSSCysteine substrates may be estimated as <2.5% of its activity for RSSG substrates as follows: No catalysis of BSA–SScysteine by TTase was observed even at an enzyme concentration 40 times that for which catalysis of BSA–SSG reduction was easily observed.

Table IV: Rates of Dethiolation for Various Protein Mixed Disulfides ± Thioredoxin[a]

substrate[b]	radiolabel assay (nmol min^{-1} mL^{-1})	thioredoxin (μM)	spectrometric assay (nmol min^{-1} mL^{-1})
papain–SSCys	12.9	5	12.1
BSA–SSCys	8.0	0.5	8.3
metHb–SSG	1.5	5	N/A[b]
metHb–SSCys	1.2	5	N/A

[a] Rate values represent the slopes of regression analyses of the linear phases of product formation vs time graphs. The values represent the mean of at least two determinations. In all cases the individual rate values deviated by 10% or less relative to the mean. Reactions were performed at 30 °C in 0.1 M potassium phosphate, pH 7.5 (except papain–SSCys; 0.1 M Tris-HCl, pH 7.5), 0.2 mM NADPH, 0.15 mM protein mixed disulfides, and 0.1 μM *E. coli* thioredoxin reductase, ± *E. coli* thioredoxin. No reactions occurred in the absence of thioredoxin. Spectrophotometric assay measures the loss of NADPH at A_{340nm}, and the radiolabel assay measures TCA-soluble counts (see Experimental Procedures). [b] N/A, not applicable; spectrophotometric assays were not performed with the hemoglobin substrates, because the heme absorbance interferes with accurate determination of A_{340} changes.

DISCUSSION

Substrate Specificity of Thioltransferase. Thioltransferases (glutaredoxins) have come to be thought of as a class of relatively nonspecific general catalysts of thiol–disulfide

exchange reactions and GSH as the reductant of the intramolecular disulfide form of TTase (Mannervik et al., 1989). The data presented in this article suggest a more restrictive substrate specificity for TTase involving a requirement for mixed disulfides containing the glutathionyl moiety, i.e., RSSG. Accordingly, only the RS half of the mixed disulfide molecule can be varied. Among the protein–SSR derivatives that we tested, TTase catalyzed formation of protein–SH from Hb–SSG, papain–SSG, and BSA–SSG (Figure 1A, Table II), but TTase did not catalyze dethiolation of Hb–SScysteine, Hb–SScysteamine, papain–SScysteine, or BSA–SScysteine (Figure 1B, Tables I and II). We specifically focused on GSH, cysteine, and cysteamine among the various nonprotein thiols that might be considered physiologically relevant components of protein–SSR mixed disulfides, because the common features of their structures provided a stringent test of specificity. Although it occurs at much lower concentrations than GSH, cysteamine in particular has been considered as a mediator of intracellular protein thiol–disulfide exchange (Ziegler, 1985), and previously reported data were interpreted by Axelsson and Mannervik (1980) to reflect TTase-catalyzed reduction of cysteamine-containing disulfides. Because the latter interpretation would mean that a cysteamine-containing disulfide could be a substrate for TTase, we examined this possibility in two ways. First, we measured the rates of GSH-dependent dethiolation of Hb–SScysteamine in the presence and absence of TTase and found no difference. Second, in additional experiments (not shown), we measured the GSH-dependent reduction of cysteamine by the GSSG reductase-coupled spectrophotometric assay and observed a distinct lag phase in the initial rate of NADP (GSSG) formation. Analogous lag phases were observed for all of the non-GS-containing mixed disulfides tested. Such lag phases have been attributed previously to buildup of the GSSG substrate for the *reporter* enzyme GSSG reductase (Axelsson et al., 1978). We observed no lag phases, however, for any of the GS-containing substrates, including GSScysteine, which was tested at concentrations as low as 5 μM. Thus, the lag phase for non-GS-containing disulfides more likely reflects a buildup of a GS-containing substrate for the *primary* enzyme TTase (e.g., Figure 2, eq 7).

Rat liver TTase exhibited the same selective catalytic characteristics as HRBC TTase; i.e., non-GS-containing disulfides were not substrates. Moreover, after this article was submitted for publication, a paper appeared (Bushweller et al., 1992) in which it was stated that the preferred substrate for *E. coli* glutaredoxin is a mixed disulfide with glutathione, but no data were provided. In contrast, we found that *E. coli* thioredoxin did not discriminate between GS-containing and non-GS-containing disulfide substrates (Table IV). Thus, the requirement for the glutathionyl moiety appears to be a general property of the thioltransferase/glutaredoxin subclass of thiol:disulfide oxidoreductase enzymes, clearly distinguishing them from the thioredoxins.

Kinetics of GSSR Dethiolation by HRBC TTase. A wide range of apparent turnover numbers (27–2200 min^{-1}; Table II) was observed for TTase-catalyzed dethiolation of GSSR substrates, and apparent K_M values also varied considerably (0.035–0.66 mM). Whereas TTase displayed the highest efficiency (V_{max}/K_M) for the small molecule GSScysteine, its catalytic efficiency was much lower for the various protein–SSG substrates. Thus the progression from GSScysteine to oxyHb–SSG covers about a 1500-fold range of efficiencies, probably reflecting steric influences. The specific difference between metHb–SSG and oxyHb–SSG likely illustrates the

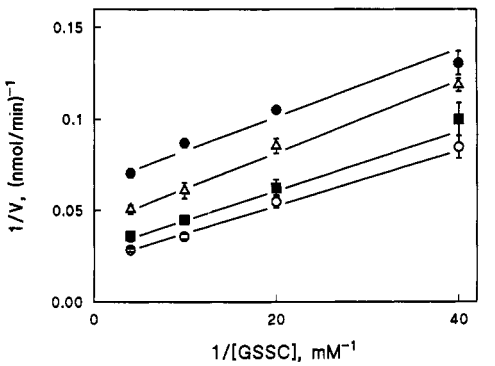

FIGURE 3: Two-substrate kinetics: dependence of TTase activity on [GSScysteine] at various GSH concentrations. Rates of GSSG formation were measured in the standard spectrophotometric assay as a function of GSScysteine concentration at several fixed GSH concentrations. Reaction mixtures in 1 mL total volume at 30 °C contained 0.1 M potassium phosphate, pH 7.5, 0.2 mM NADPH, and 2 units/mL *spinach* GSSG reductase, in the absence or presence of TTase (0.008 unit/mL). Spinach GSSG reductase was substituted for yeast GSSG reductase to maximize the specificity for GSSG. The GSScysteine concentrations are shown; the GSH concentrations were as follows: 0.5 mM (solid circles), 0.75 mM (open triangles), 1.0 mM (solid squares), and 1.5 mM (open circles). Data have been normalized to the amount of product formed per 1 mL of reaction mixture. Each data point represents the mean of four separate experiments ± SE. Where error bars are not easily apparent, they occur within the symbols.

effect of steric hindrance by the substrate protein to accessibility of the enzyme protein. Each Hb tetramer contains two β93 cysteine groups that are next to His-β92, which is ligated to the heme iron. The iron of oxyHb is in the plane of the heme and pulls His-β92 and Cys-β93 toward the interior of the protein. The iron atom of MetHb is out of the heme plane, making Cys-β93 more solvent exposed (Perutz et al., 1974). Predictably, the efficiency of dethiolation of metHb–SSG was >9-fold larger than that of oxyHb–SSG. Extending the steric consideration to BSA–SSG suggests that the cysteinyl–SS glutathionyl moiety in that case is even more accessible, because the efficiency of dethiolation is >10-fold larger than that for metHb–SSG.

Proposed Catalytic Mechanism of TTase. Thioltransferases contain an active-site dithiol tetrapeptide (Papayannopoulos et al., 1989). The first step in TTase catalysis appears to be nucleophilic attack of one of the active-site cysteines on the disulfide substrate, releasing the first thiol product. This step is thought to be facilitated by the low apparent pK_a of this cysteine (pK_a ~3.5; Gan et al., 1990; Yang & Wells, 1991a; Mieyal et al., 1991b). It has been suggested that TTase then may form an intramolecular disulfide with the second cysteine, releasing the second thiol product (Gan & Wells, 1987). Although a TTase intramolecular disulfide is chemically possible, its participation in the actual catalytic mechanism is problematic for several reasons. First, site-directed mutagenesis studies have shown equal kinetic competence of a mutant TTase in which the second cysteine was changed to serine, thereby obviating formation of an intramolecular disulfide (Yang & Wells, 1991a). Second, this mechanism would predict a ping-pong kinetic pattern. Instead, our previous two-substrate kinetic data for non-GS-containing disulfides and GSH gave sequential patterns (Mieyal et al., 1991b), suggesting either a ternary complex involving GSH, the disulfide substrate, and TTase before catalysis or a preenzymatic step in which GSH reacts with the original disulfide to form a GS-containing disulfide as the actual substrate for TTase. Our current data favor the latter interpretation (Figure 4). Accordingly, when two substrate

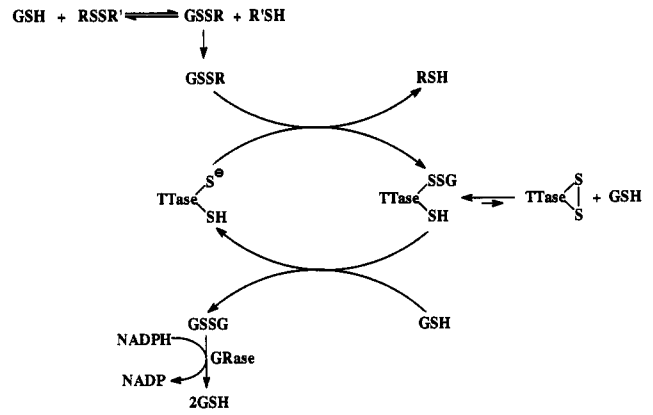

FIGURE 4: Proposed ping-pong mechanism of thioltransferase action. The central portion of this scheme depicts the simplest mechanism for TTase catalysis when a GS-containing disulfide and GSH are the cosubstrates. TTase is interconverted between the reduced thiolate form and a TTase–SSG intermediate. The upper portion of the figure shows the preenzymatic formation of GSSR consistent with a sequential kinetic pattern when a non-GS-containing substrate is present, and the lower portion depicts the coupling with GSSG reductase. On the right is shown the possible formation of the TTase intramolecular disulfide.

kinetics were performed with GS-containing disulfides (e.g., Figure 3), ping-pong patterns were observed with HRBC TTase as well as with rat liver TTase, suggesting a common mechanism for this family of enzymes. The central portion of Figure 4 depicts the simplest mechanism for TTase catalysis when a GS-containing disulfide and GSH are the cosubstrates, showing TTase being interconverted between the reduced thiolate form and a TTase–SSG intermediate. Consistent with this concept, Eriksson et al. (1974) reported that incubation of oxidized TTase with [^{35}S]GSH resulted in incorporation of radiolabel into an isolable TTase derivative, suggesting formation of [^{35}S]TTase–SSG. In this context also, Yang and Wells (1991b) offered an alternative catalytic mechanism for the mutant TTase lacking the second Cys residue, where TTase–SSG replaced the intramolecular disulfide form of TTase in their usual mechanism. These several lines of evidence support the intermediate role of TTase–SSG in the catalytic scheme; however, further study is necessary to characterize this derivative and test its kinetic competence. Since a parallel line pattern like Figure 3 does not entirely rule out an unusual form of sequential mechanism (Segel, 1975), product inhibition studies would be useful to distinguish this possibility from the more likely ping-pong mechanism that we favor.

The upper portion of Figure 4 shows the preenzymatic formation of GSSR consistent with a sequential kinetic pattern when a non-GS-containing substrate is present, and the lower portion depicts the coupling with GSSG reductase. On the right is shown the possible formation of the TTase intramolecular disulfide, but high intracellular concentrations of GSH would drive the equilibrium toward TTase–SSG and reduced TTase.

The scheme shows reaction of TTase–SSG with GSH to complete the catalytic cycle, and this would be facilitated by the presence of NADPH and GSSG reductase. Theoretically any thiol could replace GSH as the reductive regenerator of reduced TTase, because this is equivalent to the reverse reaction. Previously we demonstrated that TTase does catalyze the reverse of GSH-dependent HEDS reduction, i.e., β-mercaptoethanol-dependent GSSG reduction (Mieyal et al., 1991b). Moreover, in preliminary experiments we observed TTase-mediated dethiolation of [^{35}S]BSA–SSG when GSH (and NADPH and GSSG reductase) were replaced by cysteine (Gravina, 1993).

Physiological Implications of TTase Substrate Specificity. Although much work has been dedicated to elucidating the structure and catalytic properties of TTase, the physiological functions of TTase are not well known. It has been suggested that TTase could be involved in homeostatic maintenance of intracellular thiols and regulation of receptors and enzymes (Ziegler, 1985; Mannervik et al., 1989; Mieyal et al., 1991a,b). Thiols that have been oxidized to disulfides due to oxidative stress or during the normal catalytic cycle of some enzymes are then reduced by TTase. Pyruvate kinase, phosphofructokinase, and glutathione S-transferase are examples of proteins that are inactivated under oxidizing conditions and reactivated by TTase (Mannervik & Axelsson, 1980; Mieyal et al., 1991a; Shen et al., 1991). In most cells GSH is the major intracellular nonprotein thiol and would be expected to form the majority of any mixed disulfides. Accordingly, the formation of a glutathione mixed disulfide would serve as a tag for reduction. It is also conceivable that TTase may be a central component of a signal transduction pathway. Because protein thiols may react with TTase–SSG to form glutathionyl mixed disulfides, TTase may function to glutathionylate proteins and thereby modulate their function.

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REFERENCES

Axelsson, K., & Mannervik, B. (1980) *Biochim. Biophys. Acta 613*, 324–336.
Axelsson, K., Eriksson, S., & Mannervik, B. (1978) *Biochemistry 17*, 2978–2984.
Brocklehurst, K., & Little, G. (1973) *Biochem. J. 133*, 67–80.
Brocklehurst, K., Carlsson, J., Kierstan, M. P. J., & Crook, E. M. (1973) *Biochem. J. 133*, 573–584.
Bushweller, J. H., Aslund, F., Wuthrich, K., & Holmgren, A. (1992) *Biochemistry 31*, 9288–9293.
Caceci, M. S., & Cacheris, W. P. (1984) *Byte 9*, 340–362.
Drewes, G., & Faulstich, H. (1990) *Anal. Biochem. 188*, 109–113.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.
Eriksson, B., & Eriksson, S. A. (1967) *Acta Chem. Scand. 21*, 1304–1312.
Eriksson, S., Askelof, P., Axelsson, K., Carlberg, I., Guthenberg, C., & Mannervik, B. (1974) *Acta Chem. Scand. B28*, 922–930.
Faulstich, H., Merkler, I., Blackholm, H., & Stournaras, C. (1984) *Biochemistry 23*, 1608–1612.
Ferraiolo, B. L., Onady, G. M., & Mieyal, J. J. (1984) *Biochemistry 23*, 5528–5534.
Gan, Z.-R., & Wells, W. W. (1987) *J. Biol. Chem. 262*, 6704–6707.
Gan, Z.-R., Sardana, M. K., Jacobs, J. W., & Polokoff, M. A. (1990) *Arch. Biochem. Biophys. 282*, 110–115.
Garel, M.-C., Domenget, C., Caburi-Martin, J., Prehu, C., Galacteros, F., & Beuzard, Y. (1986) *J. Biol. Chem. 261*, 14704–14709.

Gilbert, H. F. (1990) in *Advances in Enzymology* (Meister, A., Ed.) Vol. 63, pp 69–172, John Wiley & Sons, New York.
Gravina, S. A. (1993) Characterization and Kinetic Mechanism of Thioltransferase, Ph.D. Thesis, Case Western Reserve University, Cleveland, OH.
Haas, R., & Rosenberry, T. L. (1985) *Anal. Biochem. 148*, 154–162.
Hartley, R. W., Peterson, E. A., & Sober, H. A. (1962) *Biochemistry 1*, 60–68.
Hatakeyama, M., Tanimoto, Y., & Mizoguchi, T. (1984) *J. Biochem. (Tokyo) 95*, 1811–1818.
Holmgren, A. (1985) *Methods Enzymol. 113*, 525–540.
Holmgren, A. (1989) *J. Biol. Chem. 264*, 13963–13967.
Isles, T. E., & Jocelyn, P. C. (1963) *Biochem. J. 88*, 84–88.
Kimmel, J. R., & Smith, E. L. (1954) *J. Biol. Chem. 207*, 515–531.
Mannervik, B., & Axelsson, K. (1980) *Biochem. J. 190*, 125–130.
Mannervik, B., Axelsson, K., & Larson, K. (1989) in *Coenzymes and Cofactors* (Dolphin, D., Paulson, R., & Amramovic, O., Eds.) Vol. III, Part A, Chapter 12, pp 475–516, John Wiley & Sons, New York.
Mieyal, J. J., Starke, D. W., Gravina, S. A., Dothey, C., & Chung, J. S. (1991a) *Biochemistry 30*, 6088–6097.
Mieyal, J. J., Starke, D. W., Gravina, S. A., & Hocevar, B. A. (1991b) *Biochemistry 30*, 8883–8891.
Papayannopoulos, I. A., Gan, Z.-R., Wells, W. W., & Biemann, K. (1989) *Biochem. Biophys. Res. Commun. 159*, 1448–1454.
Perutz, M. F., Fersht, A. R., Simon, S. R., & Roberts, G. C. K. (1974) *Biochemistry 13*, 2174–2186.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 623–624, John Wiley & Sons, New York.
Shen, H., Tamai, K., Satoh, K., Hatayama, I., Tsuchida, S., & Sato, K. (1991) *Arch. Biochem. Biophys. 286*, 178–182.
Sober, H. A., & Harte, R. A., Eds. (1973) *Handbook of Biochemistry (Selected Data for Molecular Biology)*, 2nd ed., p C-71, CRC Press, Cleveland, OH.
Stuchbury, T., Shipton, M., Norris, R., Malthouse, J. P. G., & Brocklehurst, K. (1975) *Biochem. J. 151*, 417–432.
Thannhauser, T. W., Konish, Y., & Scheraga, H. A. (1984) *Anal. Biochem. 138*, 181–188.
Van Kampen, E. J., & Zijlstra, W. G. (1961) *Clin. Chim. Acta 6*, 538–544.
Yang, Y., & Wells, W. W. (1991a) *J. Biol. Chem. 266*, 12759–12765.
Yang, Y., & Wells, W. W. (1991b) *J. Biol. Chem. 266*, 12766–12771.
Ziegler, D. M. (1985) *Annu. Rev. Biochem. 54*, 305–329.