pH Profiles Indicative of Rate-Limiting Nucleophilic Displacement in Thioltransferase Catalysis†

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ABSTRACT: The apparent pK_a for the active site thiol of human thioltransferase (TTase) is about 3.5, but the pH dependence of TTase-catalyzed rates of glutathione (GSH)-dependent reduction of disulfide substrates displays an inflection point near pH 8.5. The similarity of the pH−rate profile with the titration of the GSH thiol moiety suggested rate-limiting nucleophilic attack by the glutathionyl thiolate species to regenerate reduced TTase from the TTase−SSG intermediate. To test this hypothesis pH−rate profiles for TTase-catalyzed dethiolation of the glutathionyl mixed disulfide of bovine serum albumin ([^{35}S]BSA−SSG) were measured according to release of radiolabeled GS-equivalents. Various thiol compounds, whose thiol pK_a values range on both sides of the pK_a of GSH (pK_a = 8.7), were used as reducing substrates, e.g., trifluoroethanethiol (pK_a = 7.5) and 3-mercaptopropionic acid (pK_a = 10.3). The pH−rate profiles paralleled the titration of the respective thiol groups of the reducing substrates, consistent with the hypothesis. In addition, second-order rate constants (k) were determined for the nonenzymatic and TTase-catalyzed reactions of the various thiols with BSA−SSG. A simple linear free energy relationship (log k vs pK_a) was displayed for the nonenzymatic reactions. In contrast, the relationship for the enzymatic reactions revealed GSH to be different from the other thiol substrates, i.e., GSH gave a second-order rate constant greater than expected for its thiol pK_a. This result suggests a special interaction of GSH with the TTase enzyme in the transition state that enhances the nucleophilicity of GSH.

Thioltransferase (EC 1.8.4.2), also known as glutaredoxin, is a low molecular weight, cytosolic protein that belongs to the class of enzymes called thiol disulfide oxidoreductases. A characteristic feature of this class of enzymes is the presence of a pair of cysteines at the active site which can exist in reduced (dithiol) or oxidized (intramolecular disulfide or intermolecular mixed disulfide) forms. Thioltransferase has been implicated in the reduction of a wide variety of protein and low molecular weight disulfides, and the overall reaction catalyzed by this enzyme is a thiol disulfide interchange reaction involving two nucleophilic displacements (Mieyal et al., 1995; Wells et al., 1993). Typically GSH[1]-dependent TTase activity has been determined by coupling the formation of the final product GSSG to NADPH oxidation by GSSG reductase (in excess), and this spectrophotometric assay measures the rate of the overall reaction (Scheme 1). Earlier studies using this coupled assay (Axelsson & Mannervik, 1980) suggested that TTase was capable of catalyzing both the formation of the glutathionyl mixed disulfide (step 1) and its subsequent reduction by GSH (step 2), although data for catalysis of the individual steps was not reported.

Scheme 1: Two Step Reduction of Disulfides by GSH[a]

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

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

$$\text{GSSG} + \text{NADPH} \xrightleftharpoons{\text{GRase}} 2\,\text{GSH} + \text{NADP}^+ \quad (3)$$

[a] Reaction of GSH with a generic disulfide RSSR′ forms a glutathionyl mixed disulfide that is converted to GSSG in a second step. The reaction may be coupled to the NADPH/GSSG reductase system.

In order to examine TTase catalysis of step 1 or step 2 independently, we used radiolabel or HPLC assays, in the absence and in the presence of TTase, to measure GSH-dependent dethiolation of various protein mixed disulfides (protein−SSR, where R = glutathione, cysteine, or cysteam-

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[1] Abbreviations used were AMPSO, (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; BSA-SH, bovine serum albumin (*S*-carboxymethyl)-NH_2-CO-CH_2-CH_2-SH; BSA−SSG, bovine serum albumin (*S*-carboxymethyl)-NH_2-CO-CH_2-CH_2-S-S-glutathione; CAPS, (3-[cyclohexylamino]-1-propanesulfonic acid); cpm, counts per minute; Cysteine-SSG, cysteine−glutathione mixed disulfide; DMF, dimethyl formamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; GSSG, glutathione disulfide; GSSG reductase, glutathione disulfide reductase; HED, hydroxyethyl disulfide; HEPPSO, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-hydroxypropanesulfonic acid]; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; β-ME, β-mercaptoethanol; 3-MPA, 3-mercaptopropionic acid; MES, 2-[*N*-Morpholino]ethanesulfonic acid; PDI, protein disulfide isomerase, NTSP, 3,3′-dithiobis(propionic acid) *N*-hydroxysuccinimide ester; -SSCysteine, cysteine mixed disulfides; -SSG, glutathione mixed disulfides; TCA, trichloroacetic acid; TDOR, thiol disulfide oxidoreductase, TFET, trifluoroethanethiol; TTase, thioltransferase.

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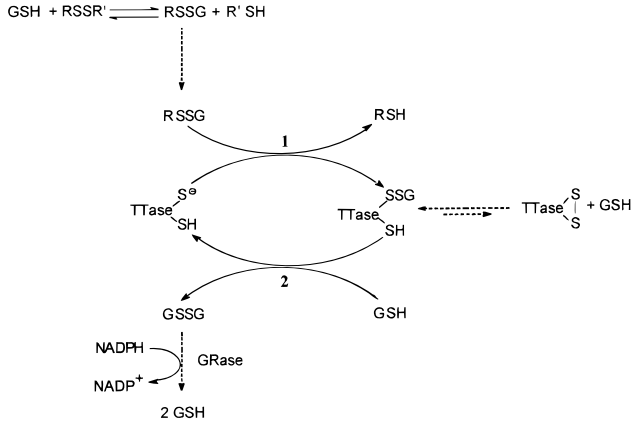
Scheme 2: Mechanism of Thioltransferase Catalysis[a]



[a] The central portion of the scheme depicts the ping-pong mechanism of TTase catalysis when a glutathionyl mixed disulfide and GSH are the substrates. In step 1 the reduced thiolate form of TTase is converted to the TTase−SSG intermediate, and the first product is released. In step 2 the TTase−thiolate is regenerated by GSH (or other thiol compounds), along with formation of GSSG (or RSSG). The upper portion of the figure shows the pre-enzymatic formation of the GSSR mixed disulfide which is the actual substrate. At the right is the possible formation of the intramolecular disulfide form of TTase that is readily converted to TTase−SSG by GSH. Coupling with GSSG reductase is shown at the bottom.

ine) (Gravina & Mieyal, 1993). In fact TTase catalyzed only the dethiolation of glutathione-containing disulfides, showing no enhancement of the rates of reaction of GSH with protein-SSCysteine (or Cysteamine) disulfides. Two substrate kinetic studies with a variety of glutathionyl mixed disulfides and GSH gave patterns consistent with a simple ping-pong mechanism involving the formation of a TTase−SSG covalent intermediate (Gravina & Mieyal, 1993).

When the overall reactions of GSH with non-GS-containing disulfide substrates were studied via the coupled assay system in the presence of TTase, the two substrate kinetic patterns indicated a sequential mechanism (Mieyal et al., 1991) consistent with a nonenzymatic formation of the GS-containing substrate before catalysis by TTase (Scheme 2, top).

In the present study glutathionylated disulfide substrates were used to investigate further aspects of the catalytic mechanism of TTase. Previous studies demonstrated that the apparent pK_a of one of the active site cysteines of TTase is 3.5 (Gan et al., 1990; Mieyal et. al., 1991; Yang & Wells, 1991a). In contrast the pH dependence of TTase-catalyzed reduction of hydroxyethyldisulfide with GSH as the second substrate gave an inflection point near pH 8.5, suggesting that the thiolate of the second substrate GSH was involved in the rate-determining step. Previously, Mannervik and Axelsson (1980) reported that other thiols like cysteamine, cysteine, dithiothreitol, and β-mercaptoethanol, among others, could substitute for GSH in supporting TTase catalysis of release of radiolabel from rat liver cytosolic sulfhydryl proteins that had been treated with radiolabeled GSSG.

We used thiol compounds with pK_as distinct from that of GSH and confirmed that they were capable of supporting TTase catalysis of reduction of the model disulfide substrate BSA−SSG, although with less efficiency than GSH. With the alternate thiol substrates we found that the pH profile of the TTase-catalyzed reaction shifted according to the thiol pK_a of the thiol substrate. These results are consistent with the hypothesis that the reaction of the thiol substrate with the TTase−SSG intermediate is rate-determining for the TTase-catalyzed dethiolation of glutathionyl mixed disulfides. Furthermore, the unusual reactivity of GSH relative to the other thiol compounds suggests a special interaction of GSH with the TTase−SSG intermediate in the transition state that enhances the nucleophilicity of GSH.

EXPERIMENTAL PROCEDURES

Materials. β-mercaptoethanol, glutathione, 3-mercaptopropionic acid, HEPPSO, AMPSO, CAPS, carboxymethyl bovine serum albumin, 3,3′-dithiobis(propionic acid) *N*-hydroxysuccinimide ester, dithiobis(nitrobenzoic acid), γ-glutamylcysteine, cysteinylglycine, and Sephadex G-25 were purchased from Sigma. Trifluoroethanethiol was obtained from Aldrich. [^{35}S]Glutathione (50−100 Ci/mmol) was obtained from NEN Dupont, and Bio-Safe cocktail was from Research Products International Corp. NADPH was purchased from Boehringer Mannheim and cysteinyl−glutathione disulfide from Toronto Research Chemicals. Thioltransferase (40−60 U/mg) was obtained either as the natural enzyme purified from human red blood cells (Mieyal et al., 1991) or as the recombinant enzyme expressed in *E. Coli* and purified (Chrestensen et al., 1995). All other chemicals were reagent grade from standard sources.

Measurement of pK_a Values. The pK_a values for the thiol moieties of β-mercaptoethanol and trifluoroethanethiol were measured conductometrically by titrating with 1 M KOH at 30 °C at an ionic strength of 0.3 M. Solution pH values were measured under argon using an Orion 701 pH meter standardized with pH 7 and pH 10 buffers prior to use. The pK_a values for the thiol moieties of GSH, γ-glutamylcysteine, and cysteinylglycine were determined from the pH-dependent changes in the respective ^{1}H-NMR signals corresponding to the methylene group bonded directly to the thiol moiety in each of these compounds (-CH_2-SH). ^{1}H-NMR spectra were recorded at ambient temperature on a Bruker AMX 270 MHz NMR spectrometer. All solutions were prepared in D_2O, and the observed pH was varied by titrating with NaOD at an ionic strength of 0.3 M that was maintained with NaCl.

Standard Coupled Assay for Thioltransferase. Spectrophotometric assay of TTase activity was determined according to Mieyal et al. (1991), except that cysteine−SSG was used as the substrate (Gravina & Mieyal, 1993) instead of *S*-sulfocysteine or HED. Rate of formation of GSSG coupled to the loss of NADPH was determined from the slope of the linear portion of the time course of decreasing A_{340nm}. The slopes for minus TTase controls were subtracted from slopes for the TTase-containing samples to determine enzyme-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}).

Radiolabel Assay for TTase Catalysis. The spectrophotometric assay for TTase activity coupled to GSSG reductase and NADPH is not suitable for studying TTase catalysis with thiol substrates other than GSH since GSSG reductase is selective for GSSG. Instead a radiolabel assay (Gravina & Mieyal, 1993) was used to measure the rate of release of TCA-soluble [^{35}S]GS-equivalents from [^{35}S]BSA−SSG, without NADPH and GSSG reductase. Enzyme-dependent deglutathionylation rates were determined from the slope of the linear time course of release of [^{35}S]GS-equivalents. The rates for minus TTase controls were subtracted from those of TTase-containing samples to obtain the TTase-catalyzed

rates. [^{35}S]BSA−SSG was incubated at 30 °C with 0.1 M potassium phosphate, pH 7.5, 0.04−0.12 units of hRBC TTase (Mieyal, et al., 1991) or recombinant hTTase (Chrestensen et al., 1995). The kinetic properties of various purified preparations of TTase were confirmed to be indistinguishable in these studies. The ionic strength was maintained at 0.3 M by addition of appropriate amounts of NaCl. The assay was initiated by the addition of GSH. Reactions were stopped at different times by adding 0.05 mL of the reaction mixture to 0.1 mL of 20% ice-cold TCA. The samples were centrifuged at 10 000 rpm in a Fisher microfuge for 2 min, then 0.1 mL of the supernatant was added to 5 mL of Bio-Safe scintillation cocktail and counted in a Beckman LS 5000 CE liquid scintillation counter. Net release of radioactivity in the supernatant was determined, and the background counts per minute were accounted for by extrapolating the linear region of time courses to zero time. To account for radioactive decay of ^{35}S, aliquots of [^{35}S]BSA−SSG were used to determine the specific radioactivity on the day of use. All rates were determined from the linear region of the corresponding time courses, and substrate concentration dependence was studied under conditions where enzyme concentration and time dependence were linear, as determined in separate experiments. It was confirmed also that the spectrophotometric and radiolabel assays of rates of deglutathionylation gave similar results.

Preparation of [^{35}S]BSA−SSG. A 5-fold molar excess of NTSP (dissolved in dimethyl formamide) was added to 2 mM of *S*-carboxymethyl BSA slowly with constant stirring at room temperature in 0.1 M sodium phosphate, pH 6.0, for 1 h. The reaction was terminated by adding a 3-fold molar excess of glycine relative to the NTSP and stirring for an additional 2 h. The modified BSA was treated with a 15-fold molar excess of β-ME to reduce the newly incorporated disulfide bond, then bubbled with argon, sealed, and kept for 6 h under gentle stirring. The excess β-ME and other small molecules were removed by Sephadex G-25 chromatography. BSA concentration was determined spectrophotometrically, using $\epsilon_{280nm} = 44$ mM^{-1} cm^{-1}. Sulfhydryl content of the albumin derivative was determined with DTNB relative to a standard curve for GSH, generated according to the method of Ellman (1959). The [SH]/[BSA] ratio of the BSA−SH was 1.00 ± 0.13 ($n = 11$).

[^{35}S]BSA−SSG was then prepared by treating the BSA-SH with a 5-fold molar excess of [^{35}S]GSSG. [^{35}S]GSSG was prepared by treating unlabeled GSSG with a trace amount of [^{35}S]GSH at pH 8.5, overnight at 4 °C. The typical specific activity of fresh preparations of [^{35}S]GSSG was in the range 0.5−0.7 mCi/mmol. The incorporation of [^{35}S]GS-equivalents into [^{35}S]BSA−SSG was followed by removing aliquots of the reaction mixture periodically, precipitating the protein with TCA, washing the pellet, and measuring the amount of radioactivity associated with the precipitated protein. After the reaction was complete, the excess [^{35}S]GSSG was separated from the [^{35}S]BSA−SSG by G-25 chromatography (0.01 M sodium phosphate, pH 7.0). The radioactive fractions coeluting with the protein were pooled and concentrated to a final concentration of 1 mM with respect to BSA. The average ratio of [^{35}S]GS/BSA for various preparations was 0.95 ± 0.15.

Two Substrate Kinetics. [^{35}S]BSA−SSG and GSH were varied independently to produce a 4 × 4 matrix, and V_{max} and K_M values were obtained by fitting the data to rectangular hyperbolic relationships. The data are presented in the figure as double reciprocal plots in order to facilitate pattern recognition.

Assay of TTase with Alternate Thiol Substrates. TTase was assayed as described under the radiolabel deglutathionylation assay, except that the reaction was initiated by alternate thiol substrates (TFET, 3-MPA, β-ME, etc.) instead of GSH, and dethiolation rates were measured at 30 °C. Fresh stock solutions of the thiol reagents were made in deoxygenated water (purged with argon) on the day of use and stored on ice. In the case of TFET both the stock solution and the reaction mixtures were sealed in reactivials with minimal head space and kept under argon. Concentrations of the thiol substrates were varied from 0.1−8 mM. Buffers used for the different pH ranges were MES (pH 4−6), HEPPSO (pH 7−8), AMPSO (pH 8−10), and CAPS (pH 10−12). The buffers were confirmed independently to give indistinguishable rates of TTase-catalyzed reactions by measuring the reaction of GSH with BSA−SSG in the different buffers within their overlapping pH ranges.

The extent of product formation ([^{35}S]GS-equivalents released) included in rate determinations was normally 10−15% of initial substrate content and never exceeded 30% of total substrate in all of the time courses reported. pK_a values were generated by fitting the data by nonlinear least-squares analysis to a modified form of the Hill equation (Markley, 1974). Correlation coefficients were calculated using a curve fitting program from Miller (1981).

RESULTS

pH Profile of TTase Catalysis. The pH profile of the overall reaction for TTase-catalyzed reduction of HED by GSH displays an inflection point near pH 8−8.5 (Yang & Wells, 1990; Mieyal et al., 1991). In contrast, the apparent pK_a of the active site thiolate (cys-22) is around pH 3.5 as determined by iodoacetamide inactivation studies (Gan & Wells, 1987; Gan et al., 1990; Mieyal et al., 1991), suggesting that the initial attack of the active site thiolate on the disulfide substrate is not rate determining (Scheme 2, step 1). The similarity of the pH profile of the overall reaction with the pK_a of GSH suggests that the rate-limiting step involves nucleophilic attack of the glutathione thiolate. The previously reported pH profiles of TTase catalysis of disulfide reduction used the nonglutathionyl substrate hydroxyethyldisulfide, so it was not clear whether the attack of GSH on the TTase−SSG intermediate (Scheme 2, step 2) was rate-limiting or whether the pH profile merely reflected the titration of GSH for the pre-enzymatic formation of the glutathionyl mixed disulfide which would then act as substrate for TTase (Scheme 2, top). We resolved this ambiguity by studying the pH−rate profiles for the GSH-dependent TTase catalysis of deglutathionylation of cysteine−SSG and BSA−SSG, because these glutathionyl mixed disulfides require no pre-enzymatic reaction with GSH. Figure 1 shows the pH−rate profiles for BSA−SSG and cysteine-SSG both with inflection points near pH 8.5, i.e., indistinguishable from that for HED. These results indicate that the nature of the disulfide substrate did not affect the pH−rate profile for TTase catalysis, suggesting that step 2 of Scheme 2 is rate determining. To test this hypothesis further GSH was replaced by other thiol compounds.

pH Profiles for BSA−SSG Dethiolation Using Alternate Thiol Substrates. Using [^{35}S]BSA−SSG as the prototype

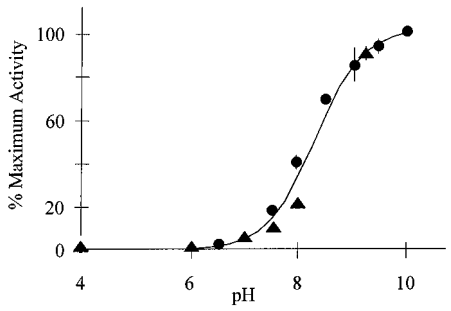


FIGURE 1: pH-rate profiles of GSH-dependent TTase catalysis of deglutathionylation of BSA−SSG and Cysteine−SSG. For cysteine−SSG the rate of formation of GSSG was measured in the absence and in the presence of TTase (0.004−0.013 μM) by the standard spectrophotometric assay with GSH at 30 °C in a final volume of 0.2 mL, except that 0.1 M potassium phosphate buffer was replaced by various buffers at 0.1 M, for the pH ranges indicated: Acetate (pH 3−4), MES (pH 4−6), HEPPSO (pH 6−7), Tricine (pH 8−9.2). The data represent TTase-mediated rates after subtracting nonenzymatic rates of GSSG formation (see Experimental Procedures). The solid triangles represent the data for cysteine−SSG. Deglutathionylation rates for BSA−SSG (solid circles) were followed at 30 °C for 2−3 min (linear phase) by measuring the release of [^{35}S]GS-equivalents in the absence and presence of TTase (0.05−0.15 μM) in various buffers at 0.08 M in 0.2 mL total volume: MES (pH 4−7), HEPPSO (pH 7−8), AMPSO (pH 8−10), and CAPS (pH 10−12). In all cases the ionic strength was adjusted to 0.3 M with NaCl, and the reactions were initiated with GSH. Rates are presented as percents of maximum activity and normalized for the TTase concentration used. Each data point represents the mean of at least two experiments plus or minus standard error (where error bars are not evident, they are within the size of the symbol).

disulfide substrate for TTase-catalysis of deglutathionylation, we studied the pH−rate profiles for the displacement of the glutathionyl moiety by thiol compounds besides GSH. Thus, TFET, β-ME, and 3-MPA, whose pK_a values are substantially different from that of GSH (Table 1), were tested as alternate thiol substrates for TTase. The pH profiles of the enzyme-catalyzed reactions showed inflection points at pH 7.0 ± 0.1 for TFET, pH 8.1 ± 0.1 for GSH, and pH 10.2 ± 0.2 for 3-MPA (Figure 2). Thus, the enzymatic pH−rate profile in each case parallels the pK_a of the thiol compound used and reflects the ionization of the thiol substrate and not any titratable groups on the enzyme (Table 1). Likewise, the pH−rate profiles for the nonenzymatic reactions of the thiol compounds with BSA−SSG reflect the pK_as of the respective thiols (Table 1).

β-Mercaptoethanol was also studied as an alternate thiol substrate because it provided the opportunity to examine the influence, if any, of the relative redox potentials of the thiol substrates. Although the redox potential of β-ME is quite different from that of 3-MPA, their pK_a values are similar (Table 1). In contrast, 3-MPA and GSH have similar redox values (K_{ox} ratio = 1.5), but their pK_as are more different. Table 1 shows that the TTase-catalyzed rate of deglutathionylation of BSA−SSG reflected the pK_a of the thiol used, regardless of the relative redox ratios. Also, β-ME and 3-MPA displayed the same concentration dependence despite their difference in redox potential (see below). Hence, relative redox potential of the thiol substrates appears not to be an important factor in the rates of TTase-catalyzed reactions.

Dependence of the TTase-Catalyzed Rate on Concentration of Thiol Substrates. At a fixed concentration of BSA−SSG and constant pH, the concentrations of several thiol substrates were varied. In each case the pH of the reaction mixtures were adjusted to the pK_a of the particular thiol compound, so that thiolate content remained at 50% of the total thiol concentration. The rate of TTase-catalyzed deglutathionylation of BSA−SSG increased as a function of GSH concentration and appeared to saturate at high GSH concentrations (Figure 3A). In contrast, the concentration dependence of the TTase-catalyzed rate with either β-ME or 3-MPA displayed a much shallower slope that remained linear (nonsaturating) over the same concentration range where the GSH-dependent rate had plateaued (Figure 3A). β-ME and 3-MPA displayed much higher nonenzymatic rates than GSH (Figure 3A, inset), which precluded determination of enzymatic deglutathionylation rates at higher concentrations of these compounds. Thus, the enzymatic turnover achievable with other thiols was low compared to the maximal turnover of TTase achieved with GSH. Therefore, the pH variation of the enzyme-catalyzed BSA−SSG dethiolation reaction was studied as a function of GSH concentration, so that a complete analysis of the relationship k_{cat}/K_M vs pH could be obtained (Figure 3B).

This graph reflects only one inflection point corresponding to the pK_a of GSH. The apparent K_M for GSH changed by 20-fold over the range pH 6−10 (i.e., 0.1 and 2.0 mM, respectively), while the k_{cat} value for the reaction changed by only 25% over the same range (k_{cat} = 600 min^{-1} at pH 6; k_{cat} = 800 min^{-1} at pH 10), indicating little if any contribution from ionization of enzyme residues.

Table 2 lists the second-order rate constants for the reactions of the various thiol compounds with BSA−SSG, all at the same pH = 9.5, in the absence and in the presence of TTase. The rate constants for the TTase-catalyzed reactions with GSH and γ-glutamylcysteine are remarkably large relative to the other thiol compounds (see Discussion).

Two Substrate Kinetic Studies for BSA−SSG and GSH. Figure 4 shows the pattern of double reciprocal plots representing variation of the TTase-catalyzed rate as a function of GSH concentration at several fixed concentrations of BSA−SSG. A set of parallel lines were obtained, consistent with previous studies (Gravina & Mieyal, 1993) and indicative of the ping-pong mechanism depicted in Scheme 2. An analogous pattern was observed when BSA−SSG concentration was varied at several fixed concentrations of GSH (data not shown). An estimate of a "true" K_M value for GSH (at saturating BSA−SSG concentration) could not be derived from the data since a secondary replot of reciprocal apparent K_M (GSH) versus reciprocal BSA−SSG concentration did not give a positive *y*-intercept (Figure 4, inset), suggesting that the apparent saturation at each fixed concentration of BSA−SSG was reflective of a limiting amount of the TTase−SSG intermediate rather than binding of GSH to the enzyme−SSG covalent complex (see Discussion).

DISCUSSION

Rate Determining Step in TTase Catalysis. On the basis of our previous characterization of the glutathionyl disulfide specificity of TTase (Gravina & Mieyal, 1993), we proposed the catalytic mechanism depicted in the center of Scheme 2. The first step is attack of the active site thiolate of TTase (apparent pK_a 3.5) on the glutathionyl mixed disulfide substrate leading to formation of the TTase−SSG intermediate and release of the first product. The second substrate GSH then reduces the TTase−SSG and regenerates the

Table 1: Apparent pK_a Values for the TTase-Catalyzed and Nonenzymatic Reduction of [^{35}S]BSA−SSG by Thiol Substrates with Different pK_a Values and Redox Properties

thiol substrates[a,c,d]			pH-rate profiles of BSA−SSG reduction[b,c,d]	
	properties			
compound	K_{ox} ratios	apparent pK_a of thiol moiety	apparent pK_a of TTase-catalyzed reaction	apparent pK_a of nonenzymatic reaction
GSH		8.7	8.1 (0.98)	8.3 (0.92)
3-MPA	GSH/3-MPA 1.5	10.3	10.2 (0.80)	10.1 (0.91)
β-ME	β-ME/3-MPA 1101	9.8 (0.92)	9.3 (0.95)	9.5 (0.95)
TFET		7.5 (0.91)	7.1 (0.96)	7.0 (0.92)

[a] Apparent pK_a values for thiolate formation for GSH and 3-MPA are reported values (Reuben & Bruice, 1976; Szajewski & Whitesides, 1980; respectively), and the GSH value was reconfirmed by NMR as described under Experimental Procedures. The pK_a values for β-ME and TFET were determined conductometrically as described under Experimental Procedures. K_{ox} ratios were calculated from reported $E°$ values. The reported $E°$ values for GSH and β-ME at pH 7.5, 30 °C, and 0.09 M ionic strength are −0.23 and −0.29 V, respectively (Mieyal et al., 1991). The reported $E°$ values for GSH and 3-MPA at pH_{obs} 7.0, 25 °C, and 0.15 M NaCl are −0.205 and −0.2 V (Rabenstein et al., 1992). [b] The rate of deglutathionylation of [^{35}S]BSA−SSG by the various thiol compounds was determined at 30 °C in the absence and presence of TTase (0.07−0.2 μM) by measuring the release of [^{35}S]GS-equivalents, as described under Experimental Procedures. All reactions were initiated by adding the thiol substrate. To set the various pH values different buffers were used at a final concentration of 0.1 M. The ionic strength in all cases was adjusted to 0.3 M with NaCl and the reactions were initiated with the thiol substrate. The pH ranges for the buffers were acetate, pH 3−5; MES, pH 4−7; HEPPSO, pH 6−8; Tricine, pH 8−9.2; AMPSO, pH 8−10; and CAPS, pH 10−12. [c] Apparent pK_a values were calculated by nonlinear least-squares fitting of the data to a modified form of the Hill equation (Markley, 1974). [d] Correlation coefficients (r^2) shown in parentheses were calculated using a curve fitting program from Miller (1981).

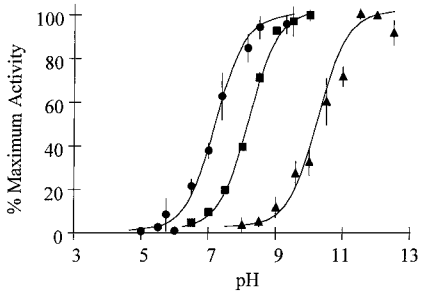


FIGURE 2: pH−rate profile for the TTase-catalyzed dethiolation of [^{35}S]BSA−SSG by TFET, GSH, and 3-MPA. TFET (1.0−8.0 mM; solid circles), GSH (0.1−0.5 mM; solid squares), and 3-MPA (0.1−1.0 mM; solid triangles) were tested at the various pH values shown with [^{35}S]BSA−SSG (0.1−0.3 mM) in a total volume of 0.2 mL. In all cases the inflection point of the pH−rate profiles were found to be independent of the specific concentrations of BSA−SSG and thiol compound. The following buffers were used: MES (pH 4−7), HEPPSO (pH 7−8), AMPSO (pH 8−10) and CAPS (pH 10−12). All buffers were 0.1 M, and ionic strength was maintained at 0.3 M with NaCl. Rates of deglutathionylation in the absence and in the presence of TTase (0.07−0.2 μM) were followed for 2−3 min according to release of radioactivity as described under Experimental Procedures. Nonenzymatic rates were subtracted from overall rates to obtain TTase-catalyzed rates. The basal activity was uniformly subtracted from the rates at different pH values and the resultant data were normalized to the maximal activity, concentration of the thiol substrate, TTase concentration, and [^{35}S]BSA−SSG concentration. Each data point represents the mean of at least four experiments plus or minus standard error (where error bars are not evident, they are within the size of the symbol). The relationships between the TTase-catalyzed rates and pH were curve fitted to a modified form of the Hill equation (Markley, 1974) in order to obtain the pH−rate profiles.

TTase thiolate (turnover) with concomitant formation of GSSG (step 2). It is expected that other thiols can replace GSH in step 2 because this reaction represents the reverse of the formation of TTase−SSG from RSSG and reduced TTase.

In a recent report (Rabenstein & Millis, 1995), NMR line broadening was used to study the rate of thiol/disulfide exchange for GSH/GSSG (and related redox pairs) in the absence and presence of TTase, and the authors cited Scheme 2 (Gravina & Mieyal, 1993) as a model for their studies. Although they suggested that formation of TTase−SSG was rate determining, the basis for their statement was not evident since the observed spectral changes do not distinguish the forward and reverse steps of the TTase-catalyzed GSH/GSSG exchange reaction. No TTase catalysis was observed for the cystine/cysteine pair, i.e., the exchange rate was not fast enough to be measured by NMR, consistent with the specificity of TTase for glutathionyl disulfides (Gravina & Mieyal, 1993). TTase catalysis was observed for γ-glutamylcysteine exchange with its symmetrical disulfide, but the rate was slower than that for GSH/GSSG (Rabenstein & Millis, 1995). This result agrees with our observation[2] that the TTase catalyzed rate of formation of [^{14}C]BSA-SS-γ-glutamylcysteine from BSA-SH and [^{14}C]γ-glutamylcysteine disulfide was much slower than that for the analogous formation of [^{35}S]BSA−SSG. This distinction is remarkable in light of the similar reactivity of GSH and γ-glutamylcysteine in the reduction of the TTase−SSG intermediate (see below).

The pH profile of the TTase-catalyzed deglutathionylation of BSA−SSG by GSH showed an inflection point around pH 8.3 (Figure 1). The similarity of this pH dependence to the titration of the GSH thiolate itself suggested that nucleophilic attack of the thiolate substrate GS^- (or RS^-) on the TTase−SSG intermediate was rate limiting for the overall reaction (step 2, Scheme 2), rather than formation of TTase−SSG (step 1), at fixed concentrations of enzyme and disulfide substrate. Using a variety of thiol compounds other than GSH to test this hypothesis, we found that the pH−rate profiles for the TTase-catalyzed reactions in all cases reflected the pK_a of the thiol compound used (Figure 2, Table 1), consistent with the hypothesis that breakdown of the TTase−SSG intermediate is rate determining.

The pH−rate profile for the TTase-catalyzed reaction shifts in parallel with the pK_a value of the thiol substrate, and each of the pH profiles with the different thiols appears to be monolithic, i.e., representative of only one ionization (Figure 2). These findings which cover a broad pH region preclude the possibility of an important contribution from an ionizable group on the enzyme because this would have appeared as a second inflection point in one of the pH profiles. This conclusion was reinforced by the k_{cat}/K_M vs pH relationship

[2] Srinivasan, U., & Mieyal, J. J. (1995), unpublished observations.

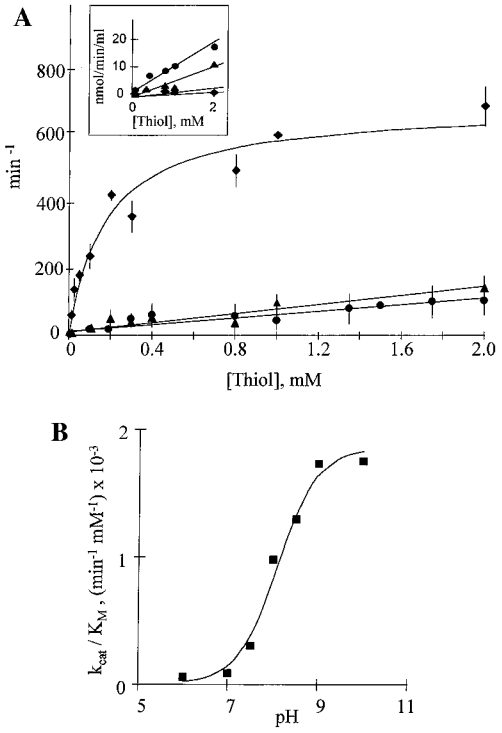


FIGURE 3: (A) Dependence of TTase catalyzed deglutathionylation of [^{35}S]BSA−SSG on the concentrations of GSH, 3-MPA, and β-ME. TTase activity (0.02−0.24 μM) was assayed by the radiolabel dethiolation assay (see Experimental Procedures). Reactions were initiated by the addition of the thiol substrate at 30 °C to a solution containing 0.1 mM BSA−SSG and 0.3 M ionic strength in 0.35 mL final volume at the pH values corresponding nearly to the respective pK_as of the different thiol substrates. The thiol compounds used were GSH (solid diamonds), β-ME (solid circles), and 3-MPA (solid triangles). The buffers used were HEPPSO at pH 8.5 for GSH, AMPSO at pH 9.5 for β-ME, and CAPS at pH 10.0 for 3-MPA. Nonenzymatic rates were measured at each concentration (inset) and subtracted from the overall rates to obtain the TTase-catalyzed rates. Data were normalized to the amount of product formed per 1 mL of reaction mixture. The TTase-catalyzed rates are presented as k_{cat} (turnover number, min^{-1}); i.e., nanomoles of GS-equivalents released per minute per nanomole of TTase. Each data point represents the mean of at least two experiments plus or minus standard error (where error bars are not evident, they are within the size of the symbol). (B) pH dependence of k_{cat}/K_M for TTase-catalyzed deglutathionylation of [^{35}S]BSA−SSG by GSH. Reactions were initiated by the addition of GSH at 30 °C to a solution containing 0.1 mM BSA−SSG in 0.25 mL of final volume at the pH values indicated. The buffers [HEPPSO (pH 6−8), AMPSO (pH 8−10)] were 0.1 M, and ionic strength was maintained at 0.3 M with NaCl. Rates of deglutathionylation in the absence and in the presence of TTase (0.09 μM) were followed for 2 min according to release of radioactivity as described under Experimental Procedures. Nonenzymatic rates were subtracted from overall rates to obtain TTase-catalyzed rates. TTase-catalyzed rates at each pH value were determined as a function of GSH concentration and the data were fitted to nonlinear rectangular hyperbolic relationships to obtain values for K_M and k_{cat}. The k_{cat}/K_M vs pH relationship was curve fitted to a modified form of the Hill equation (Markley, 1974). The K_M for the reaction changed by 20-fold over the range pH 6−10 (i.e., 0.1 and 2.0 mM, respectively), while the k_{cat} value changed by only 25% over the same range (k_{cat} = 600 min^{-1} at pH 6; k_{cat} = 800 min^{-1} at pH 10).

for the reaction with GSH which had only one inflection point and was dictated almost exclusively by changes in the K_M for GSH (Figure 3B). Flohe et al. (1972) confronted an analogous situation with glutathione peroxidase where other thiols were used to replace GSH as the substrate that turns over the enzyme−peroxide intermediate. However, in a clear contrast to TTase, the pH−rate profile for glutathione peroxidase did not shift with the pK_a of the thiol compounds used. Accordingly, the authors concluded that their pH profile was reflective of an ionization on the enzyme. The

Table 2: Second-Order Rate Constants for Release of [^{35}S]GS-Equivalents from [^{35}S]BSA−SSG by Thiol Compounds, in the Absence (RS^- + BSA−SSG) and Presence of TTase (RS^- + TTase−SSG)[a]

compound	k (M^{-1} min^{-1})	
	RS^- + BSA−SSG[b]	RS^- + TTase−SSG[c]
Trifluoroethanethiol	24 ± 7 (6)	4.1 ± 0.8 × 10^4 (6)
3-Mercaptopropionic acid	121 ± 26 (7)	2.0 ± 0.6 × 10^5 (7)
β-Mercaptoethanol	55 ± 11 (10)	0.9 ± 0.2 × 10^5 (10)
Glutathione	109 ± 40 (4)	2.2 ± 0.3 × 10^6 (4)
γ-Glutamylcysteine	93 ± 8 (4)	1.0 ± 0.5 × 10^6 (4)
Cysteinylglycine	88 ± 19 (4)	1.1 ± 0.2 × 10^5 (4)
Cysteine	89 ± 19 (6)	1.3 ± 0.1 × 10^5 (6)

[a] Rates of deglutathionylation of BSA−SSG (0.1 mM) in 0.1 M AMPSO, pH 9.5, and 0.3 M ionic strength were determined by the radiolabel dethiolation assay (Experimental Procedures). At least four different concentrations of the thiol compound were tested in each case, in the absence and in the presence of TTase (0.02−0.24 μM): TFET, 0.125−1.0 mM; 3-MPA, 0.1−1.0 mM; β-ME, 0.1−2.0 mM; GSH, 0.025−0.2 mM; γ-glutamylcysteine, 0.2−1.0 mM; cysteinylglycine, 0.2−1.0 mM; and cysteine, 0.1−1.0 mM. Nonenzymatic rates were subtracted from the overall rates to obtain the TTase-catalyzed rates. In separate experiments it was confirmed that the TTase concentrations were in the linear region of concentration dependence. Rate constants were calculated from these data, and each value represents the mean of at least four separate experiments (n, as shown) ± standard error. [b] Second order rate constants are expressed as min^{-1} M^{-1} relative to BSA−SSG concentration for the nonenzymatic reaction. [c] In the presence of TTase, rate constants are expressed as min^{-1} M^{-1} relative to TTase−SSG concentrations, assuming that all of the TTase is in the form of TTase−SSG.

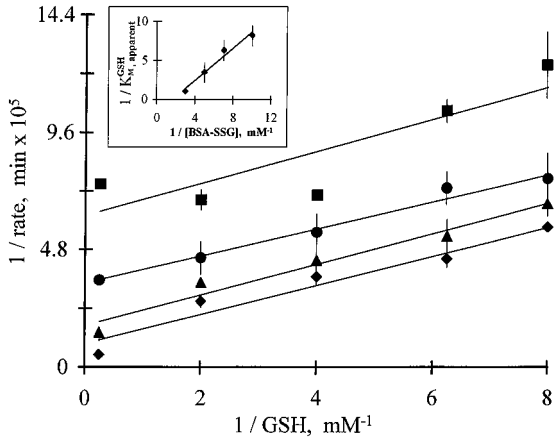


FIGURE 4: Two substrate kinetics for BSA−SSG and GSH: dependence of TTase activity on GSH concentration at various BSA−SSG concentrations. Rates of GSSG formation were measured by the standard spectrophotometric assay except that GSH and BSA−SSG concentrations were varied as shown. Reaction mixtures were 0.2 mL total volume at 30 °C, containing 0.1 M potassium phosphate, pH 7.5, 0.2 mM NADPH, and 2 units/mL yeast GSSG reductase in the absence or in the presence of TTase (0.026 μM). The BSA−SSG concentrations were as follows: 0.1 mM (solid squares), 0.14 mM (solid circles), 0.2 mM (solid triangles), 0.3 mM (solid diamonds). The TTase-catalyzed rates were calculated as k_{cat} (turnover number, min^{-1}); i.e., nanomoles of GSSG formed per minute per nanomole of TTase. Data are presented in double reciprocal format for ease of pattern recognition. Each data point represents the mean of at least two experiments plus or minus standard deviation (where error bars are not evident, they are within the size of the symbol). (Inset) Secondary plot of reciprocal of apparent K_M of GSH versus reciprocal concentration of BSA−SSG. K_M values were calculated from fitting the data to nonlinear rectangular hyperbolic relationships.

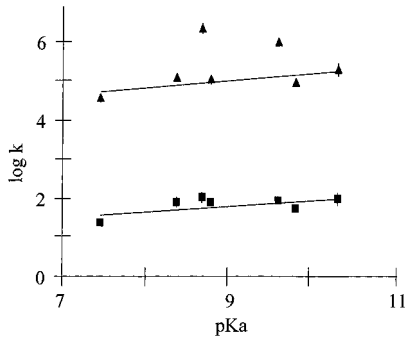


FIGURE 5: Dependence of calculated second-order rate constants on the pK_as of the thiol substrates. Rates of deglutathionylation of BSA−SSG (0.1 mM) in 0.1 M AMPSO, pH 9.5, and 0.3 M ionic strength were determined for at least four different concentrations of each of the thiol compounds (as described under Table 2), in the absence and in the presence of TTase (0.02−0.24 μM): TFET (pK_a = 7.5), cysteine (pK_a = 8.4), GSH (pK_a = 8.7), cysteinylglycine (pK_a = 8.8), γ-glutamylcysteine (pK_a = 9.6), β-ME (pK_a = 9.8), and 3-MPA (pK_a = 10.3). Rate constants were calculated from these data and expressed as min^{-1} M^{-1} relative to BSA−SSG concentration for the nonenzymatic reactions (closed squares) and as min^{-1} M^{-1} relative to TTase−SSG concentration for the enzyme-catalyzed reactions (closed triangles), assuming that all of the TTase is in the form of TTase−SSG. Apparent thiol pK_a values for GSH, cysteine, and 3-MPA are reported values (Reuben & Bruice, 1976; Szajewski & Whitesides, 1980; respectively). The pK_a for GSH was reconfirmed, and the pK_a values for γ-glutamylcysteine and cysteinylglycine were determined by NMR as described under Experimental Procedures. The pK_a values for β-ME and TFET were determined conductometrically as described under Experimental Procedures. Each data point represents the mean of at least four experiments plus or minus standard error (where error bars are not evident, they are within the size of the symbol). The two lines shown were determined by linear regression analysis of the data for the five typical thiol compounds, excluding GSH and γ-glutamylcysteine (see text).

difference in pH behavior between TTase and glutathione peroxidase suggests that the transition state for thiol-mediated breakdown of the respective covalent enyme intermediates is different.

Does TTase Have a High Affinity for Reduced GSH? The thiol concentration dependence of TTase-catalyzed dethiolation of BSA−SSG revealed a higher efficiency of TTase turnover by GSH compared to other thiol compounds (Figure 3A). At a constant concentration of BSA−SSG the rate increased with glutathione concentration until the reaction of BSA−SSG with TTase (step 1, Scheme 2) became limiting. This accounts for the saturation kinetics observed at high concentrations of GSH. Two substrate kinetic studies with BSA−SSG and GSH revealed that the apparent K_M of GSH and the apparent V_{max} displayed a linear dependence on the BSA−SSG concentration, accounting for the failure of the double reciprocal plot to extrapolate to a "true" K_M for GSH (Figure 4, inset). Similarly a "true" K_M for BSA−SSG was not observable (data not shown). Thus, initial velocity experiments provided no indication of binding of either the oxidized substrate (BSA−SSG) or the reducing substrate (GSH). Consistent with this analysis, Yang and Wells (1991b) reported that reduced [^{3}H]GSH does not bind to recombinant pig liver TTase. Furthermore, the glutathione analogs *S*-methylglutathione and *S*-nitrosoglutathione did not inhibit TTase activity at concentrations as high as 3.0 and 4.5 mM, respectively (data not shown).[2] These results argue against a specific binding site for reduced GSH in ground state complexes with TTase, contrary to previous models which assumed such GSH binding sites for related glutaredoxins (Wuthrich et al., 1994; Eklund et al., 1991).

A pattern for GSH concentration dependence similar to the present study was reported for protein disulfide isomerase, a TDOR enzyme capable of catalyzing thiol/disulfide exchange reactions and GSH-dependent reduction of a variety of proteins and nonprotein thiols and disulfides (Varandani, 1978; Gilbert, 1989). At a constant concentration of a model hexapeptide disulfide the velocity of the overall reaction continued to increase with increasing GSH concentration until the bimolecular reaction of PDI with the disulfide became rate-limiting and showed saturation by GSH. Again, all the steps involved in turnover were bimolecular with respect to PDI and GSH and there was no indication of a "true" K_M for GSH in that system.

Despite the indications of low affinity of TTase for GSH, it is expected that GSH is the physiological second substrate for the enzyme because of the relative intracellular abundance of GSH. Regarding the relative nonspecificity of the enzyme for the reducing substrate, TTase is remarkably similar to glutathione peroxidase. Both enzymes display ping-pong kinetics indicative of analogous mechanisms. Glutathione peroxidase also does not have high affinity for reduced GSH, and other thiols can substitute for GSH (Mannervik & Carlberg, 1989). Accordingly, Flohe et al. (1971) did not detect binding of [^{14}C]GSH to the reduced enzyme.

Basicity and Thiol Nucleophilicity. As seen from previous studies, thiol disulfide exchange reactions are expected to be S_N2-type reactions in which the rate is dependent chiefly on the basicity (pK_as) of the nucleophiles and the leaving groups (Gilbert, 1990), and the reactive species is the ionized thiolate (Creighton & Darby, 1993). Thus, a linear free energy relationship exists between rate of thiol disulfide exchange and thiol basicity:

$$\log k = C + \beta_{nuc} pK_a^{nuc} + \beta_c pK_a^{c} + \beta_{lg} pK_a^{lg}$$

where β_{nuc}, β_c, and β_{lg} are Brønstead coefficients for the nucleophile, central group, and leaving group in the reaction:

$$RS^{lg}S^{c}R + RS^{-\ nuc} \rightarrow RS^{nuc}S^{c}R + RS^{-\ lg}$$

Szajewski and Whitesides (1980) effectively demonstrated the correlation between basicity of the thiol nucleophile and its rate of attack on a sterically unencumbered sulfur−sulfur bond. The greater the basicity of the thiol (i.e., higher pK_a), the greater its reactivity in a thiol−disulfide exchange reaction. For the deglutathionylation of BSA−SSG by thiols, we calculated the second-order rate constants both for the nonenzymatic reaction of BSA−SSG with the various thiol compounds and for the TTase-catalyzed reactions where TTase−SSG (formed from BSA−SSG and TTase-S^-) reacts with different thiol compounds (Table 2). The nonenzymatic reaction between BSA−SSG and the different thiol compounds fit a regular linear free energy Brønstead relationship (Figure 5), as expected from the previous studies of Szajewski and Whitesides (1980).

In a given thiol−disulfide exchange reaction, the second-order rate constant for the reaction increases by a factor of 3−5 ($\cong$4) for each unit decrease in the pK_a of the leaving group thiolate (Gilbert, 1990). The difference in pK_a between the leaving group in the nonenzymatic reaction (BSA-S^-, p$K_a \cong$ 8.5) and in the TTase catalyzed reaction (TTase-S^-, p$K_a \cong$ 3.5) is about five pH units; hence a rate enhancement

of about 4^5 would be expected, i.e., approximately 1000-fold. Indeed the slope of the log k vs pK_a plot for the TTase-catalyzed reactions with the various thiols is shifted by about 3 orders of magnitude (1000-fold) relative to the corresponding plot for the nonenzymatic reaction (Figure 5). Thus, with the typical thiol compounds (other than GSH and γ-glutamylcysteine, see below) the rate enhancement by TTase reflects the relative pK_a values of BSA-S^- and TTase-S^-, and the relative nucleophilicity of the thiols follows their basicity (Figure 5). Hence, the catalytic effect of TTase is identified with the pK_a of the active site Cys-22 residue in its role as a leaving group in step 2, rather than as a nucleophile in step 1 (Scheme 2).

Although the rate constant for the nonenzymatic reaction with GSH fit with the other thiols (lower curve, Figure 5), the value for GSH in the TTase-catalyzed reaction was remarkably greater than that predicted by the slope of the upper curve in Figure 5, i.e., GSH showed a rate enhancement of about 20-fold. Thus, the nature of the interaction of the second substrate with the TTase−SSG intermediate is an important determinant in the efficiency of TTase turnover. The super activity of GSH suggests a special interaction between GSH and TTase−SSG in the transition state of the enzymatic reaction. As an initial approach to the molecular basis for this special interaction we studied the turnover of TTase−SSG by the subcomponents of GSH, namely γ-glutamylcysteine, cysteinylglycine, and cysteine. Like GSH, γ-glutamylcysteine was found to be super efficient in TTase turnover, whereas cysteinylglycine and cysteine were indistinguishable from the other thiol compounds that obeyed the Brønstead relationship (Figure 5). Thus, the γ-glutamylcysteinyl moiety of GSH appears to be primarily responsible for the special reactivity of GSH with thioltransferase. Further study is necessary to delineate the basis for the enhancement of GSH nucleophilicity by TTase.

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