Articles

Thioltransferase in Human Red Blood Cells: Purification and Properties[†,‡]

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ABSTRACT: Thioltransferase activity was identified and the enzyme purified to apparent homogeneity from human red blood cells. Activity was measured as glutathione-dependent reduction of the prototype substrate hydroxyethyl disulfide; formation of oxidized glutathione (GSSG) was coupled to NADPH oxidation by GSSG reductase (1 unit of activity = 1 μmol/min of NADPH oxidized). The thioltransferase–GSH–GSSG reductase system was shown also to catalyze the regeneration of hemoglobin from the mixed disulfide hemoglobin-S-S-glutathione (HbSSG) and to reactivate the metabolic control enzyme phosphofructokinase (PFK) after oxidation of its sulfhydryl groups. On a relative concentration basis, thioltransferase was about 1200 times more efficient than dithiothreitol in reactivation of phosphofructokinase; e.g., 500 μM DTT was required to effect the same extent of reactivation as that of 0.4 μM TTase. The GSH plus GSSG reductase system *without thioltransferase* was ineffective for reduction of HbSSG or reactivation of PFK. The average amount of thioltransferase in intact erythrocytes was calculated to be 4.6 units/g of Hb at 25 °C. This level of activity is about the same as those of other enzymes that participate in sulfhydryl maintenance in red blood cells, such as GSSG reductase and glucose-6-phosphate dehydrogenase. These results suggest a physiological role for the thioltransferase in erythrocyte sulfhydryl homeostasis. Certain properties of the human erythrocyte thioltransferase resemble those of other mammalian thioltransferase and glutaredoxin enzymes. Thus, the human erythrocyte enzyme, purified about 28 000-fold to apparent homogeneity, is a single polypeptide with a molecular weight of 11 300. Its N-terminus is blocked, it is heat stable, and it contains four cysteine residues per protein molecule. However, the human erythrocyte thioltransferase is a distinct protein based on its amino acid composition. For example, it contains no methionine residues; whereas the related mammalian enzymes described to date have at least one internal methionine residue in their largely homologous sequences.

Maintenance of the redox status of sulfhydryl groups is vital to the physiological functions of red blood cells. Thus, Hb[1] has two reactive Cys β93-SH groups at its conformationally sensitive dimer interface; modification of these SH groups alters O_2 and heme binding (Makino & Sugita, 1982). Similarly, oxidation of SH groups on PFK inactivates this critical metabolic control enzyme (Valentine, 1984) and thereby interferes with energy production in RBCs. Disulfide cross-linking of SH groups on integral membrane proteins like spectrin (Snyder et al., 1988) alters RBC permeability and deformability (Wagner et al., 1988). Depending on the nature and reversibility of these sulfhydryl modifications, they conceivably might regulate or interfere with normal erythrocyte functions. Although most cells are susceptible to oxidative injury, RBCs seem to be more at risk because of their high iron content and exposure to high oxygen tension as well as oxidative dietary constituents, environmental chemicals, and drugs.

Traditionally, GSH with GSSG reductase has been viewed as the system responsible for maintaining and regenerating protein-SH groups that are susceptible to disulfide oxidation in RBCs. Although GSH coupled to the reductase and NADPH does provide reducing equivalents, this system alone seemed insufficient for maintaining protein sulfhydryl status for several reasons: (1) as indicated above, the RBCs are confronted with unusual oxidative stress; (2) the kinetic effectiveness of GSH as a direct reducing agent for protein disulfides is limited [e.g., Creighton (1983)]; (3) thioltransferase enzymes that catalyze the GSH-dependent reduction of protein disulfides have been identified in other tissues (Mannervik & Axelsson, 1980; Hatakeyama et al., 1984; Larson et al., 1985). On the basis of the above considerations, we investigated and found thioltransferase activity in human erythrocytes.

In this article, we report the purification and properties of a thioltransferase enzyme from human erythrocytes. According to its properties the HRBC TTase belongs to the

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[‡] This article is dedicated to Robert H. Abeles on the occasion of his 65th birthday.

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[1] Abbreviations: $CSSO_3$ sodium *S*-sulfocysteine; DTNB, 2,2′-dithiobis(5-nitrobenzoate); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; G-6-PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; GSSG, oxidized glutathione; HEDS, hydroxyethyl disulfide; Hb, hemoglobin; IAA, iodoacetamide; MMTS, methyl methanethiosulfonate; MW, molecular weight; PDS, 2,2′-pyridine disulfide; PFK, phosphofructokinase; RBC(s), red blood cell(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH, sulfhydryl; HRBC TTase, human red blood cell thioltransferase.

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superfamily of thiol–disulfide oxidoreductases, including glutaredoxins and thioredoxins. Coupled to GSSG, reductase the HRBC TTase was shown to catalyze the GSH-dependent reduction of HbSSG and the reductive reactivation of disulfide-modified phosphofructokinase in vitro. Thus, this enzyme displays activities that would contribute to sulfhydryl homeostasis in red blood cells, and its level is similar to the activities of other RBC enzymes like GSSG reductase and glucose-6-phosphate dehydrogenase that participate in this physiological role.

Experimental Procedures

Materials. Prestained low molecular weight standards were obtained from Amersham or from Integrated Separation Systems. Acrylamide, bisacrylamide, Tricine, sodium dodecyl sulfate (SDS), hydroxylapatite (Bio-Gel HTP), and unstained low molecular weight standards were obtained from Bio-Rad. Phenylmethanesulfonyl fluoride (PMSF) and NADPH were obtained from Boehringer Mannheim. Thioredoxin, *Escherichia coli*, was obtained from CalBiochem. [^{14}C]Iodoacetamide (23 nCi/nmol) was obtained from ICN. Hydroxyethyl disulfide (HEDS), phosphofructokinase (from rabbit muscle), 2,2′-pyridine disulfide (PDS), methyl methanethiosulfonate (MMTS), ATP–Mg, fructose 6-phosphate, triosephosphate isomerase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, 2-mercaptoethanol, dithiothreitol, ethylenediaminetetraacetic acid (disodium), glutathione (reduced), sorbitol, Tris base, and iodoacetamide were obtained from Sigma. Sephadex G-75 and G-50, agarose-IEF, ampholytes 6–8, and phenyl-Sepharose were obtained from Pharmacia. Glutathione reductase (from yeast or from bovine intestinal mucosa) was obtained from U.S. Biochemicals and Sigma. *S*-sulfocysteine (sodium salt) was synthesized according to the procedure of Segel and Johnson (1963) and then twice recrystallized from aqueous ethanol. All other reagent grade chemicals and solvents were from standrad sources.

Standard Assay for Thioltransferase Activity. A 1-mL sample of aqueous reaction mixture contained 0.2 mM NADPH, 0.5 mM GSH, 0.1 M potassium phosphate buffer, pH 7.5, 0.4 unit of GSSG reuctase, and 2 mM hydroxyethyl disulfide. Separate concentrated stock solutions of the assay components were divided into aliquots and stored frozen (−70 °C). GSH, NADPH, potassium phosphate, and GSSG reductase were mixed, diluted with the appropriate proportions of water and thioltransferase enzyme solution (total volume 0.9 mL), and preincubated in the spectrophotometer for 5 min at 30 °C before the reaction was initiated with 0.1 mL of 20 mM HEDS. 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. In separate experiments it was confirmed that GSSG reductase was not limiting and that there was a 1:1 correspondence between GSSG production and NADPH utilization. One unit of TTase activity is defined as 1 μmol of NADPH oxidized per min (i.e., A_{340nm} for NADPH = 6.2 mM^{-1} cm^{-1}) under these standard assay conditions.[2]

Protein Assays. Measurements of protein concentrations were done routinely by the Pierce microtiter plate BCA (bicinchoninic acid) assay with reagents supplied by Pierce and the standard protocol described in the manufacturer's instruction booklet; bovine serum albumin was used as standard. Results were consistent with those from assays performed by the method of Lowry et al. (1951).

Preparation of Hemolysates. Red blood cells (RBCs) were separated by centrifugation from human blood obtained as outdated samples from the American Red Cross. The RBCs were washed at least three times with 0.9% NaCl and then lysed by adding 3 volumes of deionized water. The membranes were removed by ultrafiltration with a Millipore Pellicon tangential-flow apparatus, as described previously by Rosenberry and Scoggin (1984). The stroma-free hemolysate was adjusted to 3 mM Na_2EDTA, 1 mM NaN_3, 1 mM 2-mercaptoethanol, and 10 μM phenylmethanesulfonyl fluoride to provide antibacterial, antiproteolytic, and sulfhydryl protection, and it was diluted with water to 0.5 mM with respect to Hb concentration (assayed as cyanoferric-Hb: A_{541nm} = 44 mM^{-1} cm^{-1}; Van Kampen & Zijlstra, 1961). In separate experiments this hemolysate concentration was found to be optimal for the first step of thioltranferase purification and for quantitative analysis of TTase content, described below.

Stepwise Purification of HRBC TTase: (1) Selective Solvent-Induced Denaturation/Ultrafiltration. Stroma-free hemolysate (0.5 mM Hb, 1200 mL) at 4 °C was combined while stirring with 400 mL of solvent (ethanol/$CHCl_3$ 2:1, precooled overnight at −70 °C), and the mixture was stirred at 4 °C for 15 min. The resulting suspension was centrifuged (15000*g*, 20 min), and the supernatant water/ethanol solution was carefully removed by suction from the Hb-rich pellet and trapped $CHCl_3$ layer. The supernatant was bubbled vigorously with N_2 to drive off residual $CHCl_3$. The procedure usually was repeated 12 times, and the supernatants were combined (total volume about 14.5 L). This solution was pumped and recycled through a Pellicon tangential-flow ultrafiltration apparatus (Millipore) fitted with two PTGC filter cassettes in order to concentrate proteins with MW ≥ 10000 into a smaller volume without changing the concentration of solvent or small solutes. When the retentate was diminished to 2.4 L, it was diluted with deionized water to 4 L to decrease the ethanol content to <10%, and then the Pellicon process was continued until the recovered volume, including a water rinse of the apparatus, was 400 mL. The 400-mL solution was concentrated further to ≤80 mL via an Amicon stirred-cell ultrafiltration device fitted with a YM5 membrane, under 55 psi N_2. The concentrated solution was centrifuged (15000*g*, 20 min) to remove any additional precipitate.

(2) Sephadex G-75 Gel Filtration Chromatography. The preparation from step 1 was applied to a 5 cm × 75 cm (1.5 L) Sephadex G-75 column equilibrated and eluted with 10 mM sodium phosphate, pH 7.5/1 mM 2-mercaptoethanol at 4 °C. Fractions (21.5 mL) were collected at 50–60 mL/h. The TTase activity eluted as a symmetrical peak after residual Hb and other higher molecular weight proteins had eluted. Fractions with an activity to protein ratio [(units/mL)/A_{280nm}] ≥ 0.4 were pooled (about 350 mL) and concentrated to about 20 mL. This and all subsequent concentration steps were done by ultrafiltration (Amicon YM5). Besides removing larger proteins, the G-75 chromatography also removed ethanol.

(3) Selective Heat-Induced Denaturation. The concentrated sample after G-75 chromatography was partitioned into 2-mL aliquots in glass test tubes in ice. These aliquots were transferred to a hot water bath (60 °C) and shaken intermittently for 2 min and then replaced in ice for 5 min. The heat-treated chilled aliquots were recombined and centrifuged

[2] We have also monitored thioltransferase activity routinely with *S*-sulfocysteine (sodium cysteinethiosulfate) as the non-glutathione substrate. In this case, the standard assay was the same as described above except that 2 mM HEDS was replaced by 1 mM $CSSO_3$ and the reactions were run at 25 °C. Under these conditions a given amount of enzyme displayed essentially the same rate of NADPH oxidation in the coupled assay as in the HEDS assay.

(15000*g*, 20 min). The resultant supernatant contained >90% of the TTase activity before heat treatment. The degree of purification at this stage varied with the purity of the post-G-75 sample.

(*4*) *Phenyl-Sepharose Hydrophobic Chromatography.* To enhance hydrophobic interactions this procedure was conducted at room temperature. The concentrated TTase preparation after heat treatment was adjusted to 1 M $(NH_4)_2SO_4$ and applied to a column (1.5 × 11 cm; 20 mL) of phenyl-Sepharose equilibrated with 1 M $(NH_4)_2SO_4$/10 mM sodium phosphate, pH 7.0. The column was washed with 2 column volumes (40 mL) of 0.8 M $(NH_4)_2SO_4$ (diluted from 1 M with 10 mM sodium phosphate, pH 7.5); then eluted with a 60-mL (3 column volumes) linear gradient set up as follows: chamber 1 (stirred and connected to the column) contained 30 mL of 0.8 M $(NH_4)_2SO_4$; chamber 2 (connected to chamber 1) contained 30 mL of 10 mM sodium phosphate buffer, pH 7.5. After half of the gradient was completed [i.e., 0.4 M $(NH_4)_2SO_4$], the solution in chamber 2 was adjusted to 10% ethylene glycol, so that the second half of the gradient proceeded 0.4–0.0 M in $(NH_4)_2SO_4$ and 0–10% in ethylene glycol. Finally, the column was washed with 1.5 volumes (30 mL) of 10% ethylene glycol. Throughout the procedure, fractions (2 mL) were collected at an average flow rate of 30 mL/h. Most of the TTase activity was eluted in the second half of the $(NH_4)_2SO_4$ gradient; fractions containing ≥ 30 (units/mL)/A_{280nm} were pooled (about 25 mL), cooled in ice, and concentrated to about 2 mL.

(*5*) *Sephadex G-50 Gel Filtration Chromatography.* The concentrated sample from phenyl-Sepharose chromatography was applied to a 1.5 cm × 25 cm (45 mL) Sephadex G-50 column equilibrated and eluted with 10 mM sodium phosphate, pH 7.2, at 4 °C, in order to remove $(NH_4)_2SO_4$, ethylene glycol, and residual large proteins from the TTase. Fractions (1 mL) were collected at an average rate of 10 mL/h. TTase activity eluted near 60% column volume; fractions with specific activity ≥ 30 (units/mL)/A_{280nm} were pooled (about 20 mL) and concentrated to about 2 mL.

(*6*) *Hydroxylapatite Chromatography.* The concentrated sample after G-50 chromatography was adjusted to 20 mM HEDS (TTase substrate), and this was applied to a 1.5 × 6 cm (10 mL) hydroxylapatite column equilibrated with sodium phosphate buffer, pH 7.2. The column was eluted with the same buffer at an average flow rate of 12 mL/h, and 1-mL fractions were collected. The TTase activity eluted near the solvent front just after a peak of contaminating protein. Fractions with nearly equal activity:A_{280nm} ratios were pooled and concentrated to <0.4 mL. The concentrated solution was adjusted to 20% glycerol and stored at 4 °C.

Assay of the Intrinsic Activity of Thioltransferase in Red Blood Cells. Hemolysates were prepared from RBCs and adjusted to 0.5 mM with respect to [Hb] as described above, and their TTase activities were determined by an internal standard, paired comparison method that controlled for loss of activity due to sample processing to remove interfering Hb. Specific incremental microliter amounts of previously isolated standard HRBC TTase (1.3 units/mL) were added either to 1-mL portions of water (standard curve) or to 1-mL portions of hemolysate (sample curve). The hemolysate samples were cooled to 4 °C in 1.5-mL Eppendorf tubes, and 0.3 mL of solvent (ethanol/chloroform 2:1 at −70 °C) was added to each; they were mixed by vortex and allowed to stand at 4 °C for 5 min before centrifugation (5 min, top speed, Fisher Model 59A microcentrifuge). Aliquots (0.1 mL) of the sample supernatants and of the standards were assayed for TTase activity as described above, and the data in each case were plotted as units per milliliter of TTase activity versus amount of standard TTase added. The *y*-intercept of the standard curve was at the origin. The *y*-intercept of the sample curve was indicative of the intrinsic TTase activity in the hemolysate, and it was corrected for recovery by multiplying it by the ratio of slopes (standard/sample). The average recovery for analyses of 11 different hemolysate preparations was 53 ± 3%.

Carboxyamidomethylation of HRBC TTase and E. coli Thioredoxin. Samples (0.1 mL) of purified HRBC TTase or *E. coli* thioredoxin were added to 0.9 mL of a buffer containing 6.7 M guanidine hydrochloride, 0.56 M Tris-HCl, pH 8.8, 5.6 mM EDTA, and 56 mM DTT. The resulting solution was mixed and heated to 95 °C for 30 min. Then 1.0 mL of 200 mM [^{14}C]-radiolabeled or unlabeled iodoacetamide in 6 M guanidine hydrochloride/0.5 M Tris-HCl, pH 8.8/5 mM EDTA was added and the mixture was maintained at 95 °C for an additional 30 min in the dark. The samples were then dialyzed overnight against two 1-L portions of 5 mM sodium phosphate buffer, pH 7.5.

Cyanogen Bromide Treatment and Analysis of HRBC TTase and E. coli Thioredoxin. Thioltransferase (2 μg) or *E. coli* thioredoxin (4 μg), both previously carboxyamidomethylated (as above), were diluted to 0.025 mL and added to 0.1 mL of 88% formic acid. Controls received no further additions, whereas excess cyanogen bromide (≥2 grains) was added to the samples, and all the mixtures were incubated overnight at room temperature in a sealed container purged with nitrogen and shielded from light. All mixtures were then evaporated under vacuum, reconstituted with 0.12 mL of 5 mM sodium phosphate, pH 7.5, and reevaporated; then 5 μL of gel sample buffer (20% glycerol, 8% SDS, 100 mM Tris-HCl, pH 6.8, and 0.2% bromophenol blue) was added to each. These samples were applied to a 16.5%T, 3%C Tricine SDS–PAGE gel and electrophoresed according to the method of Schagger and von Jagow (1987).

Determination of Molecular Weight via Calibrated Sephadex G-75 Chromatography. Samples of purified HRBC TTase were mixed with proteins of known molecular weight (see legend to Figure 3) and applied to a Sephadex G-75 column (400-mL column volume) preequilibrated and eluted with 10 mM potassium phosphate, pH 7.5. Fractions (5.5 mL) were collected at a rate of about 0.4 mL/min, and A_{280nm} was measured. Elution volumes were calculated from the A_{280nm} peaks in the chromatogram. The plot of elution volume vs log MW was linear. TTase elution volume was determined from the peak in a plot of elution volume vs TTase activity, and TTase MW was determined by interpolation.

Amino Acid Analysis and N-terminal Sequence Analysis. Samples of HRBC TTase and *E. coli* thioredoxin, a positive control of known amino acid composition, were hydrolyzed in 6 N HCl under N_2 at 115 °C for 16 h and analyzed on a Beckman 119CL amino acid analyzer with the standard three-buffer system as described by Haas and Rosenberry (1985). A standard mixture of the amino acids (20 nmol of each) was analyzed concurrently to provide the basis for quantitation. Cysteine content was determined in separate experiments in which HRBC TTase and *E. coli* thioredoxin were treated with [^{14}C]IAA under stringent reducing and denaturing conditions (described above), then dialyzed, hydrolyzed, and analyzed for radioactivity and amino acid composition. The radioactivity was found exclusively at the position of elution of (carboxymethyl)cysteine, and the number of Cys residues per protein molecule was calculated by ratio to the numbers of Lys and Arg residues per molecule; this

Table I: Representative Purification of Thioltransferase from Human Erythrocytes[a]

purification stage	total volume (mL)	total protein[b] (mg)	total activity[b] (units)	specific activity (unit(s)/mg)	yield (%)	purification (fold)
hemolysate	14400	501100	2160	0.004	100.0	
solvent treatment	75	8,138	1062	0.13	49.2	33
Sephadex G-75	21	216	749	3.5	34.7	870
heat treatment	19	188	686	3.7	31.8	920
phenyl-Sepharose	1.6	10.7	465	43	21.5	10700
Sephadex G-50	1.8	6.8	283	42	13.1	10500
hydroxylapatite	1.3	1.13	127	112	5.9	28000

[a] Each step in the purification scheme is described under Experimental Procedures corresponding to the designations above. [b] The values for protein and activity represent the mean of at least five determinations for each of the samples. The standard error in every case was ≤5%. The numbers for activity (units = micromoles of NADPH per minute) and protein (milligrams relative to BSA) presented above correspond to the state of the enzyme preparation immediately preceding the subsequent step, i.e., after the active fractions were pooled and concentrated by ultrafiltration.

anlaysis gave the expected 2 Cys residues for thioredoxin. Tryptophan content was also determined separately for HRBC TTase compared to *E. coli* thioredoxin (2 Trp). In this case samples of each protein were dissolved in 6 M guanidine hydrochloride, pH 6.5, and A_{280nm} measurements were used to quantitate Trp content, as described previously (Edelhoch, 1967). N-Terminal sequence analysis was performed on a sample of HRBC TTase submitted to pulse gas-phase sequencing on an Applied Biosystems 477A/120A automated protein sequencer, as described previously (Shively et al., 1986).

Preparation of Hemoglobin–Glutathione Mixed Disulfide (HbSSG). Human Hb was purified and converted to the cyanoferric form with $K_3Fe(CN)_6$ and KCN as described previously (Ferraiolo et al., 1984) in order to stabilize the heme moiety in a state that is resistant to redox changes. Then, the accessible cysteine residues were converted to mixed disulfides by treating the Hb stepwise with 2,2'-pyridine disulfide and then GSH, as described by Garel et al. (1986). The HbSSG was purified by preparative isoelectric focusing (pH 6.7–7.7) in agarose/Sephadex G-200SF as described by Pharmacia (1982).

Assay of Reduction of HbSSG by the HRBC TTase System. Reduction of HbSSG to Hb-SH was monitored by analytical thin-layer agarose isoelectric focusing over the pH range 6–8 (Pharmacia, 1982). For each assay an Isolabs TCP-2 apparatus was operated at 10 W for 45 min at room temperature. Prior to analysis, reactions were carried out for designated periods at 25 °C in a 10-μL total volume containing 2.5 μM HbSSG, 10 mM potassium phosphate, pH 7.5, 2 mM GSH, 0.2 mM NADPH, and 0.01 unit of GSSG reductase in the absence or presence of HRBC TTase (0.006 units). Reduction was measured as a shift of relative band intensity from pH 6.8 (p*I* of HbSSG) to pH 6.9 (p*I* of Hb-SH). The relative intensities were quantitated by densitometry at 610 nm, after staining the gels with Coomassie blue.

Partial Purification of HRBC Phosphofructokinase. Human erythrocyte phosphofructokinase was partially purified from 400 mL of hemolysate (100 mL of packed RBCs plus 300 mL of buffer containing 5 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM ATP–Mg), essentially according to the first three steps of the method of Staal et al. (1972), whereby PFK was selectively bound to DEAE and separated from unbound Hb. After batchwise elution, the PFK was precipitated with $(NH_4)_2SO_4$. The pellet was resuspended in buffer containing 5 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 1 mM ATP–Mg, and aliquots were stored at 4 °C. Prior to use each aliquot (0.5 mL) was adjusted to 10 mM DTT and stirred for 5 min; then the DTT and $(NH_4)_2SO_4$ were removed by Sephadex G-25 chromatography (0.5 × 8 cm column equilibrated and eluted with 5 mM potassium phosphate, pH 7.5/1 mM EDTA/1 mM ATP–Mg). The eluent fractions were tested for PFK activity and DTT content (using DTNB); active fractions free of DTT were pooled. These PFK stock solutions, which retained their activity for at least 1 week at 4 °C, were used in the protection and reactivation assays described below.

Standard Phosphofructokinase Assay. Mixtures (6 mL) containing 0.2 M Tris-HCl, pH 8.0, 1 mM EDTA, 40 mM $MgCl_2$, 0.4 mM NADH, 4 mM fructose 6-phosphate, and 4 mM ATP–Mg were prepared and frozen (−70 °C). At the time of assay these were thawed and diluted 1:2 with water. Then 2.4 units of a-glycerophosphate dehydrogenase, 28 units of triosephosphate isomerase, and 4 units of aldolase were added. A total of 1 mL of this complete assay mixture was transferred to each cuvette, and a microliter quantity of PFK stock solution was added to initiate each reaction. Absorbance changes at 340 nm were monitored as a function of time, and rates were calculated from the linear portions of these plots. One unit of PFK activity is defined as 1 μmol/min of fructose 6-phosphate converted to 3-phosphoglycerate in the coupled assay system, i.e., 2 μmol/min of NADH oxidized ($2 \times 6.2A_{340nm}$/min).

Results

Purification of HRBC TTase. Table I displays a typical purification of HRBC TTase from crude hemolysate. As shown, usually four key steps in this scheme provided the major increments in purification, namely the solvent treatment and the G-75, phenyl-Sepharose, and hydroxylapatite chromatography steps. Although specific activity was not enhanced markedly by the heat step or the Sephadex G-50 step, they were required to remove specific contaminants to achieve optimal purification in subsequent procedures.

The selective solvent denaturation procedure generally removed >99% of the Hb while retaining ≥50% of the thioltransferase activity and thereby served as an effective first step in the purification. Other first-stage methods, such as anionic- or cationic-exchange chromatography, that have been used for isolation of other RBC enzymes were not useful because the pH and ionic strength dependence of TTase and Hb binding and elution were too similar. Ammonium sulfate precipitation was an ineffective method for concentrating the TTase activity (less than 50% of the activity of partially purified TTase could be recovered in the precipitate even at 95% ammonium sulfate saturation). Therefore, all concentration steps were performed by ultrafiltration, which routinely retained >90% of the TTase activity (see Experimental Procedures). Sephadex G-75 chromatography was very effective early in the purification, because it separated the bulk of the large proteins from the smaller TTase (see below). We discovered the relatively hydrophobic nature of the HRBC TTase by its behavior on reverse-phase chromatography (Figure 1). At this stage of purification, the majority of contaminating proteins were

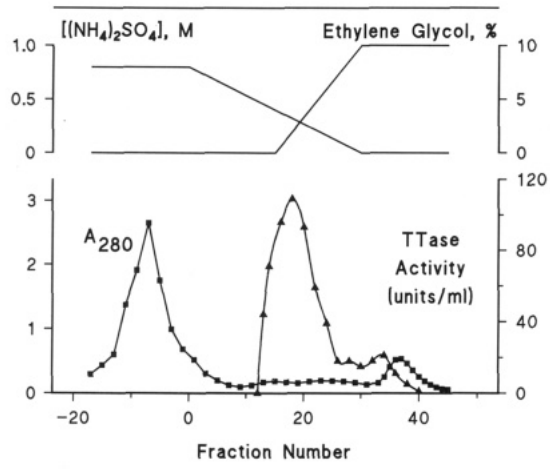

FIGURE 1: Phenyl-Sepharose hydrophobic chromatography of partially purified HRBC thioltransferase. The chromatographic protocol is described under Experimental Procedures. The gradients of ammonium sulfate (left scale; decreasing gradient) and ethylene glycol (right scale; increasing gradient) are diagrammed at the top. The elution of protein (bottom) was monitored as A_{280nm} of individual fractions (■); left scale), and TTase activity of fractions (▲); right scale) was assayed by the standard method (Experimental Procedures). Fractions 11–21 were pooled for further purification.

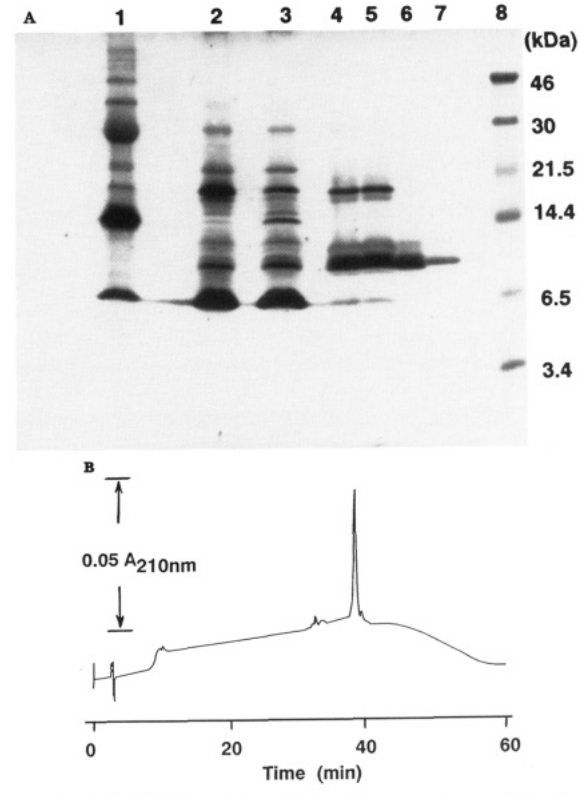

FIGURE 2: (A) SDS–PAGE analysis of progressive purification of HRBC TTase. Concentrated samples of the TTase preparations after each step of purification and after carboxyamidomethylation of the purified protein were applied to a 16.5%T/3%C/Tricine/SDS–PAGE gel and developed as described under Experimental Procedures. Lane 1: 65 μg of postsolvent treatment (step 1); lane 2: 64 μg of post-Sephadex G-75 (step 2); lane 3: 61 μg of postheat treatment (step 3), lane 4: 11 μg of post-phenyl-Sepharose (step 4); lane 5: 11 μg of post-Sephadex G-50 (step 5); lane 6: 4 μg of post-hydroxylapatite (step 6); lane 7: 5 μg of carboxyamidomethylated preparation; lane 8: 5 μL of prestained low MW standards. (B) HPLC analysis of purified HRBC TTase. An aliquot (10 μL; 9 μg) of the concentrated TTase preparation after hydroxylapatite chromatography was injected onto a Vydak C-18 HPLC column (300-Å, 5-μm spheres; 250 × 4.6 mm) and eluted at 1 mL/min with a linear 1%/min gradient of acetonitrile (0–60%) in 0.1% trifluoroacetic acid. Elution of protein was detected at 210 nm (0.2 *A* full scale; 1 mm/min chart speed). The tracing shown was expanded 4-fold vertically, as indicated.

relatively hydrophilic and eluted from the phenyl-Sepharose column in the 0.8 M ammonium sulfate wash, whereas the TTase was retained; it eluted from the resin when the positions of the gradients corresponded to about 0.3 M ammonium sulfate and about 3% ethylene glycol. Initiation of the ethylene glycol gradient at the midpoint of the ammonium sulfate gradient (before the TTase began to elute) effected a relatively narrow and symmetrical elution of TTase activity (Figure 1). The final purification was achieved by hydroxylapatite chromatography. The column was equilibrated and eluted with the same phosphate buffer, and no change in concentration of pH of the buffer was required to elute the TTase slightly after the solvent front, which contained a major protein band. The progressive removal of contaminating proteins is shown by SDS–PAGE analysis of samples from each stage of the purification scheme (Figure 2A). Lane 6 displays the post-hydroxylapatite preparation that was loaded onto the gel after the standard treatment with SDS and 3 mM DTT in boiling water for 5 min. The band for the HRBC TTase appeared diffuse or heterogeneous. This unusual appearance was seen in the preceding samples as well. Lane 7, which shows a single discrete band, represents the same preparation of HRBC TTase after the cysteine residues had been carboxyamidomethylated by treatment with IAA under denaturing conditions (see Experimental Procedures). The appearance of a single discrete band on SDS–PAGE was observed also when the HRBC TTase preparation was simply pretreated with a higher concentration of DTT, i.e., 50 mM (data not shown). These results suggest that the diffuse appearance (lane 6) is probably due to incomplete reduction of the cysteine residues or reoxidation during electrophoresis. Densitometric analysis of lane 7 indicated that this HRBC TTase preparation was at least 95% pure. This estimate was corroborated independently by HPLC analysis (Figure 2B). An aliquot of the same sample that is shown in lane 6 on the gel (Figure 2A) was chromatographed on a C-18 reverse-phase column. Despite the unusual appearance on SDS–PAGE, only one major entity is observed by HPLC analysis. The purification procedure as depicted in Table I has been carried out five times with comparable results. The post-hydroxylapatite preparations were concentrated by ultrafiltration, adjusted to 20% in glycerol and stored at 4 °C. Under these conditions there has been no loss of activity for at least 5 months.[3]

Determination of Molecular Weight of HRBC TTase. The mobility of TTase on SDS–PAGE (lane 7, Figure 2A) gave an estimated molecular weight of 11 300 relative to standard proteins (lane 8). This size estimate was found to be representative also of the native protein according to calibrated Sephadex G-75 chromatography (Figure 3). Thus, the active enzyme appears to be a single 11.3-kDa polypeptide chain.

Amino Acid Composition of HRBC TTase. Table II displays the amino acid composition of hydrolyzed HRBC TTase. The values are considered accurate to ±1 integer. Reported compositions of related thioltransferase and glutaredoxin enzymes from other tissues and species are included for comparison (Table II). All of these enzymes contain four cyteine residues, in contrast to *E. coli* glutaredoxin, which has only two cysteines (Holmgren, 1989). Consistent with its behavior on the phenyl-Sepharose column (Figure 1), HRBC TTase contains a high proportion of hydrophobic amino acids

[3] As much as 50% of the activity of a pure preparation of thioltransferase was lost rapidly, however, if a dilute solution of the enzyme was adjusted to 20% glycerol before concentration.

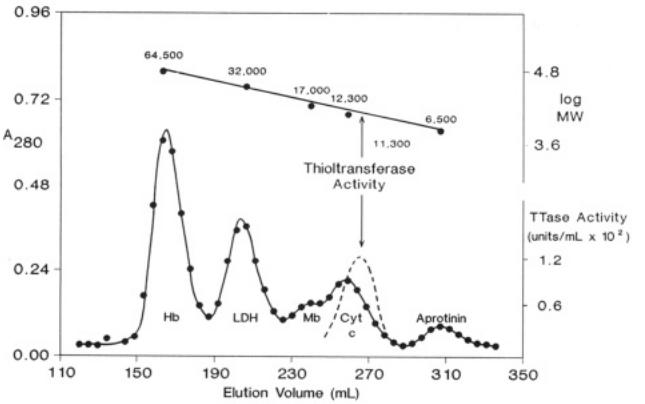

FIGURE 3: Calibrated Sephadex G-75 chromatography of HRBC TTase. Partially purified HRBC TTase (0.5 units) was mixed in 10 mM potassium phosphate, pH 7.5, with the following proteins as MW markers (as indicated on the figure): hemoglobin (30 nmol), porcine lactate dehydrogenase (10 units of isozymes 1–5), myoglobin (2 mg), cytochrome *c* (3 mg), and bovine aprotinin (3.5 mg). The sample was applied to the G-75 column and eluted and analyzed as described under Experimental Procedures.

Table II: Amino Acid Composition of HRBC TTase Compared to Other TTase and Glutaredoxin (GRx) Proteins[a]

amino acid	HRBC TTase	pig liver TTase[c]	rat liver TTase[b]	rabbit calf thymus GRx[c]	marrow GRx[c]
Asx	(7.87) 8	6	7	6	6
Thr	(6.50) 7	9	7	7	6
Ser	(4.11) 4	4	4	3	5
Glx	(14.4) 14	16	14	16	16
Pro	(8.20) 8	5	4	5	5
Gly	(7.80) 8	8	7	9	8
Ala	(6.54) 7	5	5	6	6
Cys	(3.96) 4	4	4	4	4
Val	(6.69) 7	7	5	8	7
Met	(0.05) 0	1	1	2	3
Ile	(6.19) 6	7	7	7	7
Leu	(10.4) 10	12	10	12	12
Tyr	(1.99) 2	1	2	2	2
Phe	(3.82) 4	6	5	5	5
His	(1.70) 2	1	1	1	0
Lys	(5.75) 6	8	7	7	9
Arg	(4.88) 5	5	4	5	5
Trp	(1.59) 2	0		0	0

[a] The listing for HRBC TTase represents the best estimate of integral numbers for the individual amino acids based on their mol % composition as analyzed by HPLC in comparison with a concurrent HPLC analysis of a known mixture of the amino acids (see Experimental Procedures). Calculated values were normalized and rounded to integers according to the estimated molecular weight of 11 3000 for HRBC TTase. The value for Trp was estimated from A_{280nm} of the purified protein; Cys content was determined by concurrent radioactivity analysis of a sample that was treated with [^{14}C]iodoacetamide as described under Experimental Procedures. [b] Values reported by Gan and Wells (1986). [c] Values reported by Papayannopoulos et al. (1989).

(>35%), and this seems to be characteristic of the related proteins as well (Table II).

The overall compositional analysis, however, distinguishes HRBC TTase from these other proteins. Pig liver TTase, calf thymus glutaredoxin, and rabbit bone marrow glutaredoxin have amino acid compositions and sequences quite similar to one another, including no Trp residues and at least one Met residue that is coincident at position 89 of their sequences (Papayannopoulos et al., 1989). In contrast, HRBC TTase contains two Trp and no Met residues. The absence of a methionine in a similar position in the HRBC TTase protein was corroborated by treating purified HRBC TTase and *E. coli* thioredoxin (as a positive control) with CNBr. As expected, the thioredoxin yielded two peptide fragments from cleavage adjacent to its single Met residue, whereas HRBC TTase was unaffected (data not shown).

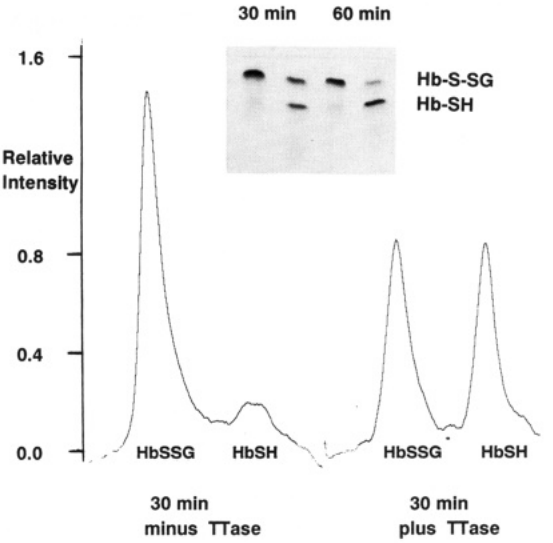

FIGURE 4: HRBC TTase-catalyzed reduction of HbSSG. As described under Experimental Procedures, HbSSG was incubated with GSH, NADPH, and GSSG reductase in the absence and presence of HRBC TTase, and the progress of the reaction was measured by the isoelectric focusing band-shift assay. The gel segment shown above represents preincubation times of 30 and 60 min. The tracings represent the output of the Shimatsu CS-930 densitometer for the "30-min" bands.

Thioltransferase Content in Red Blood Cells. A first consideration in the examination of the potential function(s) of HRBC TTase was to determine the amount of thioltransferase activity in human RBCs. We adapted the ethanol/chloroform Hb-precipitation procedure (see Experimental Procedures) for quantitative analysis of TTase content and found an average of 1.2 units of TTase/mL of RBCs [4.6 ± 0.3 units/g of Hb (n = 11)].[4] This value is similar to the activity of other RBC enzymes that participate in sulfhydryl homeostasis (see Discussion). According to the estimated MW of 11 300 (Figure 3) and specific activity for pure HRBC TTase (110 units/mg; Table I), this amount of activity corresponds to a concentration of about 1 μM TTase in human RBCs.

Reduction of Hemoglobin-S-S-glutathione. To initiate an investigation into putative physiological roles of the erythrocyte thioltransferase, we examined its ability to catalyze the reduction of disulfide-modified Hb in the presence of 2 mM GSH and GSSG reductase (Figure 4). The enzyme system with HRBC TTase present showed a distinct advantage in converting HbSSG to Hb-SH.

Protection of Phosphofructokinase from Disulfide Modification. Another potential target of oxidative stress in red blood cells is the metabolic control enzyme phosphofructokinase that is inactivated by sulfhydryl modification. To simulate this situation, we studied the inactivation of PFK by a number of sulfhydryl thiolating agents. We then tested the relative ability of GSH alone or in combination with HRBC TTase to protect PFK and/or to reactivate it.

Figure 5 illustrates the deactivation of PFK by pyridine disulfide (0.25 mM) after 1 min of exposure in the absence or in the presence of GSH and NADPH + GSSG reductase ± TTase. Conditions were chosen so that nearly complete

[4] Units are defined as 1 μmol of product/min on the basis of coupled assays with NAD(P)H. Units per milliliter of RBC was converted to the conventional representation of units per gram of Hb by the factor 0.26 g of Hb/mL of RBC. Except for the thioltransferase activity that we determined directly, the activities for the other RBC enzymes are taken from Beutler et al. (1977).

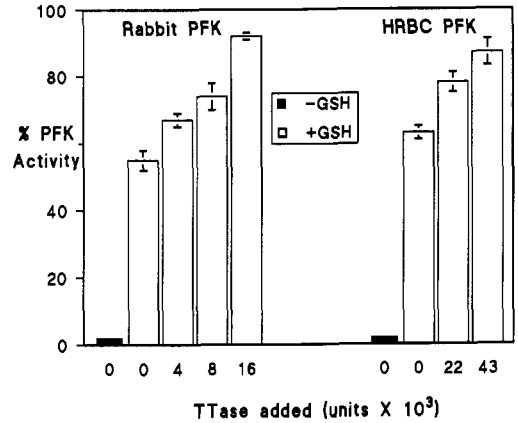

FIGURE 5: Protective Effects of GSH and HRBC Thioltransferase against pyridine disulfide inactivation of phosphofructokinase. PFK from rabbit muscle or HRBC PFK (0.1 unit) was incubated at 30 °C for 1 min in a 40-μL total volume of 0.1 M potassium phosphate containing 0.25 mM PDS in the absence or presence of [(2 mM GSH, 0.2 mM NADPH, and GSSG reductase (0.2 unit)) ± TTase (amounts indicated)]. Aliquots (2 μL) were transferred to 1 mL of standard PFK assay mixture at the end of each incubation. PFK activity was measured spectrophotometrically according to the disappearance of NADH in the coupled assay system described under Experimental Procedures. Each bar represents the mean of three to six separate experiments ± standard error.

inactivation occurred rapidly in the absence of protective agents. Both rabbit muscle PFK (left) and HRBC PFK (right) were tested. As shown, GSH (2 mM) along with GSSG reductase and NADPH did provide substantial protection within this time frame, probably by serving as a direct reductant of the PDS. Progressively more protection of PFK was provided, however, when increasing amounts of HRBC TTase were present. After 10 min of exposure to PDS the samples without TTase showed greater losses of PFK activity (data not shown). The highest concentration of TTase used was about 1 μM, which is near its estimated physiological level in RBCs (see above). Similar results were observed with MMTS as the PFK-inactivating agent (data not shown). Because protection by TTase might relate to catalysis of reduction of the PDS or MMTS as well as to reactivation of modified PFK or both, separate experiments were conducted to assess PFK reactivation.

Reactivation of PFK by TTase but not by GSH Alone. Figure 6 further delineates the action of HRBC thioltransferase. Again in the presence of a physiological concentration of GSH (2 mM), HRBC PFK was inactivated by MMTS in a concentration-dependent manner. But in this case HRBC TTase was added later to test for reactivation of the modified PFK. As shown, HRBC TTase reactivated PFK in a concentration-dependent manner. Adding more GSH (up to 2 mM additional), after the inactivation, had no effect on the residual PFK activity. Moreover, in separate experiments (not shown), we found that PDS-deactivated PFK, reisolated from excess inhibitor by gel filtration, could be reactivated by TTase plus GSH or by dithiothreitol, but not by GSH alone even up to 8 mM (data not shown). Figure 6B displays the relative concentration dependence of reactivation of MMTS-deactivated PFK by TTase and DTT. According to these data, thioltransferase was about 1200 times more efficient than dithiothreitol in reactivation of phosphofructokinase; e.g., 500 μM DTT was required to effect the same extent of PFK reactivation as that of 0.4 μM TTase.

Discussion

Characteristics of Human Erythrocyte Thioltransferase

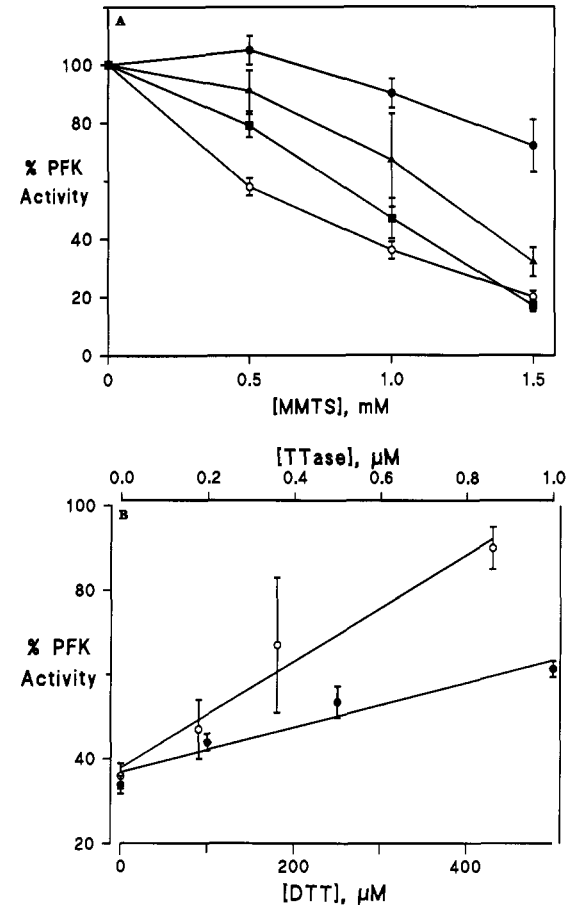

FIGURE 6: (A) Reactivation of MMTS-inactivated phosphofructokinase by HRBC thioltransferase. HRBC PFK (0.1 unit) was incubated at 25 °C with the indicated concentrations of MMTS (0.5–1.5 mM) in a 40-μL total volume containing 10 mM potassium phosphate, 2 mM GSH, 0.2 mM NADPH, and 0.2 unit GSSG reductase. After 7 min, HRBC TTase was added to all but one of the reaction mixtures as indicated, and the mixtures were incubated for an additional 20 min: (○) no TTase added; (■) 0.009 unit of TTase added; (▲) 0.018 unit of TTase; (●) 0.043 unit of TTase. Aliquots were transferred to the standard PFK assay mixture and the PFK activity was measured spectrophotometrically (see Experimental Procedures). Each data point represents the mean of four separate experiments ± standard error. (B) Concentration dependence of reactivation of phosphofructokinase by HRBC thioltransferase and DTT. The upper line (○) represents a replot of the data from panel A at 1 mM MMTS, showing the linear dependence of PFK reactivation on HRBC TTase concentration [units of TTase activity were converted to micromolar concentration (nanomoles per milliliter) by dividing units per milliliter TTase by the average specific activity of the pure TTase (8×10^{-5} units/ng) and by the mass of TTase (11 300 ng/nmol). The lower line (●) represents separate experiments with an identical 1 mM MMTS-deactivated PFK preparation in which DTT was added at the concentrations indicated instead of TTase.

Compared to Related Enzymes. We identified and isolated an enzyme from human red blood cells that has the characteristics of a thiol–disulfide oxidoreductase (EC 1.8.4.1). This class of enzymes that are found throughout nature includes thioltransferases, glutaredoxins, thioredoxins, and protein disulfide isomerases (Ziegler, 1985; Holmgren, 1989; Mannervik et al., 1989); the common features among them are an active-site pair of cysteine residues and the ability to catalyze reduction of disulfides. These enzymes have been reported to catalyze a variety of reactions besides disulfide interchange, including such apparently diverse reactions as reduction of vitamin K derivatives (Silverman & Nandi, 1988) and deiodination of iodothyronine (Goswami & Rosenberg, 1985). Specific physiological functions have not been established as yet for the individual types of thiol–disulfide oxidoreductases,

although thioredoxin and glutaredoxin have generally been linked to reduction of ribonucleotide reductase, and protein disulfide isomerase to protein folding. Protein disulfide isomerase is distinguished from the others by its larger size (MW = 57000) and localization to the endoplasmic reticulum. Among the cytosolic 11–12-kDa proteins, thioredoxin is distinguished from glutaredoxin and TTase by coupling to thioredoxin reductase rather than to GSH and GSSG reductase. Whether or not TTase and glutaredoxin enzymes are distinguishable in some way remains to be elucidated.

The human erythrocyte thioltransferase closely resembles the mammalian TTases and glutaredoxins. Thus, HRBC TTase is an 11-kDa cytosolic protein that requires GSH and GSSG reductase to support its catalytic role in the reduction of the prototype substrates hydroxyethyl disulfide and *S*-sulfocysteine. In its reduced form HRBC TTase is inactivated by IAA, and this is prevented by pretreatment with oxidized substrates (Mieyal et al., 1991). This behavior is characteristic of the dicysteine nature of the thiol–disulfide oxidoreductases. Like other thioltransferases, glutaredoxins, and thioredoxins, HRBC TTase is a heat-stable protein that can be warmed to 60–65 °C for several minutes without losing significant activity. This suggests that its global conformation may resemble that of these other proteins that contain extensive β structure (Holmgren, 1989). Mammalian glutaredoxin and TTase enzymes differ from their *E. coli* counterparts in possessing an additional pair of cysteine residues (four Cys rather than two) and a blocked N-terminal amino acid. These features are true also for the HRBC enzyme, and its overall amino acid composition is similar to the related mammalian enzymes.

Despite many similarities, there are distinct differences that distinguish HRBC TTase from the analogous proteins isolated from various other tissues and species (Table II). In particular, HRBC TTase lacks a Met residue that occurs at a common position in the amino acid sequences of pig liver thioltransferase and the calf thymus and rabbit bone marrow glutaredoxins, all of which are highly homologous (Papayannopoulos et al., 1989). This feature of its amino acid composition also distinguishes HRBC TTase from human thioredoxin (Forman-Kay et al., 1989). Whether the lack of an internal methionine residue in the HRBC TTase protein has any functional significance or represents a tissue variation or a species variation requires further study. TTase activity appears to be broadly distributed throughout the tissues of mammalian species as shown by assays of crude preparations of various tissues of cow (Hatakeyama et al., 1984) and pig (Gan & Wells, 1988). Tischler and Allen (1985) reported differences in V_{max} and K_M for a number of substrates in tests with crude preparations of TTase from a variety of rat tissues, suggesting tissue-specific variation in TTase. None of these previous studies reported activity in RBCs; however, we have detected TTase activity in bovine RBCs.[5] Although evidence for TTase activity in human kidney was reported recently (Pacifici et al., 1989), to out knowledge placental TTase is the only other human TTase that has been purified and studied (Larson et al., 1985; Wells et al., 1990). The limited information reported about the characteristics of the placental enzyme, however, gave its molecular weight as 6000 (Larson, et al., 1985) rather than 11000–12000 like HRBC TTase and analogous TTases and glutaredoxins from other species; however, the human placental enzyme displayed catalytic activities similar to those of other mammalian TTases (Larson et al., 1985; Wells et al., 1990), and it was reported to cross-react with antibodies against calf thymus glutaredoxin (Mannervik et al. 1989). Further study is necessary to delineate the basis for structural and functional differences among the TTase-like proteins from various tissues and species.

Potential Functions of HRBC Thioltransferase/Role in Sulfhydryl Homeostasis. It has been commonly thought and presented in textbooks that the mechanism for maintenance of SH groups on intracellular proteins in red blood cells involves the spontaneous reaction of GSH with oxidized protein sulfhydryls, coupled to GSSG reductase, although the possibility of an enzymatic (TTase-like) catalysis of the GSH-dependent reaction has been mentioned in previously published discussions (Mannervik & Eriksson, 1974; Kosower et al, 1977; Valentine et al., 1987). On the basis of an hypothesis that it may play an important role in sulfhydryl homeostasis, we set out to determine whether thioltransferase activity existed in RBCs. Finding the enzyme, we then sought a link between its catalytic activity and potential function by testing the action of HRBC TTase on certain substrates that might be formed in the RBCs under oxidative stress.

HRBC TTase-Mediated Reduction of HbSSG. We chose to study HbSSG as a TTase substrate because this structurally altered form of Hb that may form spontaneously in RBCs is also functionally impaired; i.e., it has a higher O_2 affinity than Hb and a lower Bohr effect (Garel et al., 1986). HRBC TTase turned out to be an effective catalyst of the GSH-dependent reduction of HbSSG, whereas the GSH and GSSG reductase system without TTase was ineffective (Figure 5). The turnover of methemoglobin in RBCs by methemoglobin reductase usually maintains a very low steady-state level of this non-O_2-binding form of Hb. Similarly, it is conceivable that thioltransferase is responsible for maintaining the low steady-state level of HbSSG. Further study is necessary to characterize the kinetics of the HbSSG–TTase reaction and to ascertain the influence of Hb ligation state on the reversible formation of HbSSG in order to more fully test the physiological role of TTase in this regard.[6]

Protection and Reactivation of Phosphofructokinase. A further test of the concept of HRBC TTase as a potential homeostatic sulfhydryl repair enzyme was directed at the glycolytic control enzyme PFK, which is susceptible to inactivation by sulfhydryl oxidation. In this case, the GSH and GSSG reductase system without TTase decreased the potency of pyridine disulfide as an inactivator of PFK (Figure 5), but the direct action of GSH was not effective in the rapid reactivation of oxidized HRBC PFK (Figure 6A). In contrast, the HRBC TTase-catalyzed reaction was found to be >1200 times more efficient than DTT in mediating reactivation of PFK (Figure 6B). These data are consistent with what has been reported regarding the relative effectiveness of GSH in reduction of protein disulfides in other contexts (Creighton, 1983). The ability of TTase-like enzymes to catalyze reversible disulfide modification of enzymes and receptors has been reviewed in the context of a potential regulatory role (Ziegler, 1985), and a number of recent studies have shown that thioredoxin systems as well as TTase systems are capable of reactivating disulfide-modified enzymes (Axelsson & Mannervik, 1983; Park & Thomas, 1988; Flamigni et al., 1989).

Proposed Role of Thioltransferase in RBC Function. TTase along with GSSG reductase and glucose-6-phosphate dehydrogenase may play a pivotal role in RBC sulfhydryl homeostasis by protecting SH groups on enzymes, Hb, and proteins important for membrane integrity, especially under

[5] H. El Messeery and J. J. Mieyal, unpublished observations.

[6] S. A. Gravina and J. J. Mieyal, work in progress.

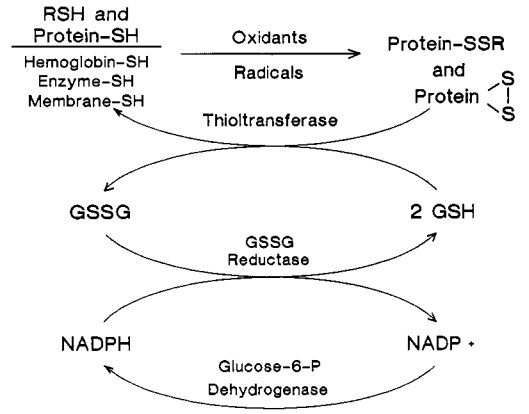

FIGURE 7: Scheme of sulfhydryl homeostasis in red blood cells under oxidative stress. Pictured is the interconversion of sulfhydryl and disulfide forms of important functional proteins of the RBCs under oxidative stress. Efficient turnover of the protein disulfides is shown to be mediated by thioltransferase, in conjunction with GSSG reductase and glucose-6-phosphate dehydrogenase.

conditions of oxidative stress; this concept is depicted in Figure 7. In this context it is interesting to compare the content of thioltransferase in human red blood cells to that of a number of other enzymes that are involved in sulfhydryl homeostasis. The level of HRBC TTase activity (4.6 units/g of Hb at 25 °C)[4] is about the same as GSSG reductase (3.9 units/g of Hb at 25 °C) to which it is coupled and glucose-6-phosphate dehydrogenase (4.6 units/g of Hb at 25 °C), the source of NADPH for the reductase (see Figure 7). After our study was completed, Wells et al. (1990) reported that protein disulfide isomerase and certain mammalian TTases can catalyze the reduction of oxidized vitamin C. Although they suggested that this action may fit into an overall scheme of cellular defense against oxidative stress whereby ascorbate would insert as an additional electron carrier beyond GSH and serve as the direct reductant of oxidized cellular components, we favor the simpler scheme proposed above for the physiology of the RBCs (Figure 7).

Other Conceivable Roles of RBC TTase. The RBCs may also serve as a circulating defense system that contributes to the protection of other tissues, and TTase would contribute to this function. In this context, Toth et al. (1984) reported that intact human RBCs decreased H_2O_2-mediated injury to isolated rat lungs and cultured bovine pulmonary cells. Although reductive repair processes appear to be the most likely function of HRBC TTase, other physiological roles involving regulation of RBC function are conceivable. For example, one might speculate that TTase could participate in modulating membrane permeability and/or O_2 exchange by promoting redox-dependent association/dissociation of Hb with the cytoplasmic domain of band 3 transport protein via thiol–disulfide interchange. Formation of the Hb–band 3 complex was found to be dependent on O_2 concentration, and it altered the conformation of the band 3 protein (Salhany & Cassoly, 1989).

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A Hypervariable Region of P450IIC5 Confers Progesterone 21-Hydroxylase Activity to P450IIC1†

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ABSTRACT: Cytochrome P450IIC5 is a hepatic progesterone 21-hydroxylase while the 95% identical P450IIC4 has a >10-fold higher K_m for progesterone 21-hydroxylation and the 74% identical P450IIC1 does not hydroxylate progesterone at detectable rates. Previous work demonstrated that the apparent K_m of P450IIC4 for progesterone 21-hydroxylation can be markedly improved by replacing a valine at position 113 with an alanine which is present at this position in P450IIC5. In the present studies, a single point mutation in cytochrome P450IIC1 that changed valine at position 113 to alanine conferred progesterone 21-hydroxylase activity to this enzyme. Although the catalytic activity was less than that of P450IIC5, these results indicate the residue 113 plays a critical role in the determination of the substrate/product selectivity in subfamily IIC P450s. By alignment with the sequence of P450cam, the segment of the polypeptide, residues 95–123, containing residue 113 corresponds to a substrate-contacting loop in the bacterial enzyme. The region containing residue 113, which is highly variable among family II P450s, may also be a substrate-contacting loop in the mammalian cytochromes P450. The exchange of this hypervariable region of cytochrome P450IIC1, residues 95–123, with that of P450IIC5 enhanced the 21-hydroxylase activity of the cells transfected with this chimera to levels similar to those of cells transfected with the plasmid encoding P450IIC5. Kinetic analysis of microsomes isolated from the transfected cells showed that the apparent K_m for progesterone 21-hydroxylation of the chimera was indistinguishable from that of P450IIC5. This suggests that despite their low amino acid similarity with P450cam, the eukaryotic P450 enzymes share some of the functional organization of the bacterial enzyme and that variation in this region is one of the mechanisms by which the eukaryotic cytochromes P450 have derived their multisubstrate specificity.

The evolution of the enzymes of the cytochrome P450 (P450)[1] superfamily has led to a multitude of catalytic functions (Nebert & Gonzalez, 1987). Highly similar isozymes frequently metabolize different substrates, and a single isozyme is often active in the metabolism of numerous structurally diverse substrates. This divergence in function seems to be independent of the divergence in primary structure in that some closely related forms are catalytically distinct and more distantly related forms catalyze the same reaction with similar efficiency. For example, cytochrome P450IIC5 is

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[1] The generic term "P450" is used to indicate a cytochrome P-450. Individual forms of P450 are designated according to the uniform system of nomenclature described by Nebert et al. (1991) with the following exceptions: The common name P450cam is used for P450CI. P450IIA4 and P450IIA5 are designated as $P450_{15\alpha}$ and $P450_{coh}$ to distinguish the steroid 15α-hydroxylase and the coumarin 7-hydroxylase, respectively (Lindberg & Negishi, 1989). Mutations are designated by indicating the one-letter abbreviation for the residue that was replaced, its position in the sequence, and the one-letter designation of the new residue in the indicated order.

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