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# Purification and Characterization of Cytoplasmic Thioltransferase (Glutathione:Disulfide Oxidoreductase) from Rat Liver†

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ABSTRACT: An enzyme catalyzing thiol-disulfide interchange of glutathione and disulfides and the reaction between glutathione and thiosulfate esters has been purified 40 000-fold from rat liver cytosol. The enzyme, named thioltransferase (Askelöf, P., Axelsson, K., Eriksson, S., & Mannervik, B. (1974) *FEBS Lett. 38,* 263-267), was homogeneous in several electrophoretic systems, had an isoelectric point at pH 9.6, and contained 8.6% (w/w) carbohydrate. The catalytic activity had a distinct optimum at pH 7.5. A series of substrates was tested at a constant glutathione level; the $k_{cat}$ values (at 4 mM glutathione) were all in the range of about $10^4$ $min^{-1}$. The substrates included mixed disulfides of glutathione, other low-molecular-weight disulfides, *S*-sulfocysteine and *S*-sulfoglutathione, and peptide disulfides such as insulin, oxytocin, ribonuclease, and the mixed disulfide of glutathione and egg-white lysozyme. The enzymatic reaction was inhibited by an excess of glutathione (>4 mM).

Glutathione is involved in a large variety of chemical reactions in the living cell, which include detoxification of oxidative and electrophilic reagents, maintenance of the proper redox state of sulfhydryl/disulfide groups, and specific reactions in cellular metabolism (e.g., isomerizations) in which glutathione serves as a coenzyme (see Flohé et al., 1974; Arias & Jakoby, 1976). However, the function of glutathione as a reductant in the scission of sulfur-sulfur bonds (in disulfides, RSSR, and thiosulfate esters, $RSSO_3^-$)

$$RSSR + 2GSH \rightleftharpoons 2RSH + GSSG$$

$$RSSO_3^- + 2GSH \rightleftharpoons RSH + HSO_3^- + GSSG$$

has received relatively little attention, in spite of the fact that this is the simplest process known for general reduction of sulfur-sulfur bonds (Mannervik & Eriksson, 1974). The biological importance of such reactions is related to the fact that low-molecular-weight thiols such as cysteine, homocysteine, and coenzyme A are used in reduced form, and several reactions occurring in aerobic cells will rapidly oxidize these thiols to disulfides. Also sulfhydryl groups in polypeptide chains may undergo oxidation to disulfides with concurrent change of their biological activities. A substantial fraction of disulfides formed by intracellular oxidation of sulfhydryl groups may be expected to be mixed disulfides of glutathione, because this compound is usually the most abundant thiol available in the cell. Even if the reduction of disulfides may result in a low steady-state level in normal cells, such mixed disulfides of low-molecular-weight thiols as well as proteins have been identified (see Mannervik & Eriksson, 1974). The maintenance of thiols in the reduced form may consequently to a large extent be related to the reduction of mixed disulfides of glutathione. The metabolism of naturally occurring thiosulfate esters, such as *S*-sulfoglutathione and *S*-sulfocysteine (Mannervik et al., 1974), and disulfide-containing peptides, such as insulin, may also be expected to require glutathione for reduction of sulfur-sulfur bonds.

An enzyme catalyzing thiol-disulfide interchange was first described by Racker (1955). Such enzyme activities have subsequently been found by several investigators (see Mannervik & Eriksson, 1974). Of the activities studied with low-molecular-weight substrates, an enzyme from bovine kidney (Chang & Wilken, 1966) and an enzyme from yeast (Nagai & Black, 1968) have been characterized in some detail. The most important reductant for the reaction in vivo is probably glutathione owing to its high concentration, but other *S*-nucleophiles can also serve as substrates for the enzyme from rat liver (Eriksson & Mannervik, 1970b). The essence of the reaction catalyzed is transfer of a thiol group (alkane sulfenyl group), and the name of "thioltransferase" has therefore been suggested for this class of enzymes (Askelöf et al., 1974) instead of the erroneous name of transhydrogenase commonly used.

In the present investigation the cytoplasmic thioltransferase found in rat liver (Eriksson & Mannervik, 1970a) has been purified extensively and characterized. Some results of this study have been reported previously (Axelsson et al., 1976).

## Materials and Methods

*Materials.* Coenzyme A, L-cystine, cystamine, GSH, L-homocystine, NADPH, oxytocin, bovine pancreatic ribonu-

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clease, and bovine trypsin were obtained from Sigma. Glutathione reductase from yeast and GSSG were from Boehringer-Mannheim. *O*-Benzoylthiamin propyl disulfide was a gift from Dr. Kohno, Tanabe Seiyaku Co., Osaka. Egg-white lysozyme was from Serva Feinbiochemica and insulin from Vitrum AB, Stockholm. Sephadex and Sepharose media for chromatography were obtained from Pharmacia and controlled-pore glass beads (CPG-10) from Electro-Nucleonics, Inc. CM-cellulose and hydroxylapatite were from Whatman Biochemicals and ampholytes for isoelectric focusing from LKB.

The following substrates were synthesized as described in the references indicated: the mixed disulfide of coenzyme A and GSH (Eriksson, 1966); the mixed disulfide of L-cysteine and GSH (Eriksson & Eriksson, 1967); the mixed disulfide of lysozyme and GSH (Axelsson & Mannervik, 1975); *S*-sulfocysteine (Segel & Johnson, 1963); *S*-sulfoglutathione (Eriksson & Rundfelt, 1968). The mixed disulfide of cysteamine and GSH was prepared by treating GSH with an excess of cystamine followed by purification by ion-exchange chromatography. All substrates synthesized were homogeneous when analyzed by electrophoresis.

*Methods.* Protein concentration was calculated from the absorbance at 260 and 280 nm (Kalckar, 1947) or determined by the procedure of Bradford (1976). The carbohydrate content was determined as described by Nowotny (1969). Sodium dodecyl sulfate and conventional polyacrylamide gel electrophoreses were performed in tubes of 2-mm internal diameter by standard techniques. In tests for homogeneity of the purified enzyme, about 10 $\mu$g of protein was applied per tube. Isoelectric focusing was performed in gel rods. The gels were prepared as described by Vesterberg (1973), and the gradient covered the range of pH 7.5 to 11. The gels were prerun for 20 min before application of the samples (about 40 $\mu$g of protein per tube).

The concentrations of trypsin, ribonuclease, insulin, and the mixed disulfide of lysozyme and glutathione were determined spectrophotometrically by measuring the absorbance at 280 nm. The concentrations of the other disulfides and the thiosulfate esters used as substrates were determined enzymatically in the assay system described below by use of purified thioltransferase and about 30 $\mu$M substrate.

The concentration of GSH used in kinetic measurements was determined by the method of Ellman (1959).

*Assay of Thioltransferase Activity.* The standard assay was based on the formation of GSSG from GSH and *S*-sulfocysteine ($CySSO_3^-$) and the subsequent reduction of GSSG by NADPH in the presence of glutathione reductase (Eriksson et al., 1974a):

$$CySSO_3^- + GSH \rightleftharpoons CySSG + HSO_3^-$$

$$CySSG + GSH \rightleftharpoons GSSG + CySH$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The oxidation of NADPH is monitored spectrophotometrically at 340 nm and, in the presence of a saturating concentration of glutathione reductase, this reaction is a direct measure of GSSG formation. The steady-state velocity, which was reached within 2 min, was used as a measure of the activity. A unit of thioltransferase activity was defined as the amount of enzyme catalyzing the formation of 1 $\mu$mol of GSSG per min (30 °C) in the following system: 0.14 M sodium phosphate (pH 7.6), 1 mM EDTA, 0.1 mM NADPH, 0.5 mM GSH, 2 units of glutathione reductase, and 3 mM *S*-sulfocysteine. The enzymatic reaction was determined by subtraction of the spontaneous reaction, measured in the absence of thioltransferase. Use of *S*-sulfocysteine as a substrate instead of, for example, the mixed disulfide of cysteine and glutathione (CySSG) is advantageous because the spontaneous reaction of the former is slow both on an absolute scale and relative to the enzymatic reaction as compared with many alternative substrates (cf. Table II).

*Kinetic Studies.* Kinetic studies were carried out in the standard assay system using variable concentrations of glutathione and disulfides (or thiosulfate esters). The measurements were made at 30 °C on an Aminco DW-2 UV/VIS spectrophotometer. The enzymatic velocities ($v$) were plotted vs. $v$/[substrate] to assess conformity to the Michaelis–Menten equation. All curves in which the GSH concentration was constant and the donor substrate varied appeared to be Michaelian. The kinetic constants were in these cases determined by use of the plot of Eisenthal & Cornish-Bowden (1974), because the error structure of the data was unknown and the subtraction of the spontaneous velocity might introduce additional variance to the data set as compared with the error structure of systems previously investigated in our laboratory (Askelöf et al., 1976). When the GSH concentration was varied, curve-fitting by the least-squares method was used (cf. Jakobson et al., 1977), because the data were non-Michaelian and could not be analyzed by use of the plot of Eisenthal & Cornish-Bowden.

*Purification of Thioltransferase.* Step 1: Preparation of Rat Liver Supernatant. The livers from 50 male specific-pathogen-free Sprague-Dawley rats (200–250 g) were homogenized in a blender in 0.25 M ice-cold sucrose and diluted with sucrose to a concentration of 10% (w/v). The homogenate was centrifuged for 45 min at 19 200$g$. The pellets were suspended in a small volume of 0.25 M sucrose and the suspension was centrifuged as above. The supernatant fractions were pooled, adjusted to pH 5.5 with cold 0.2 M acetic acid, and centrifuged for 60 min at 19 200$g$. The final supernatant fraction (about 2 L) was adjusted to pH 8 with cold 50 mM NaOH and used for the further purification. All subsequent operations were carried out at 4 °C.

Step 2: Chromatography on DEAE-Sephadex A-50. The supernatant from step 1 was diluted with an equal volume of water and passed through a column (9 × 20 cm) of DEAE-Sephadex A-50 which was previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The enzyme was not bound to the gel and it was eluted with the same buffer.

Step 3: Chromatography on CM-Cellulose I. The pooled effluent from step 2 was adjusted to pH 6.1 with 0.2 M acetic acid and applied to a column (9 cm × 15 cm) of CM-cellulose equilibrated with 10 mM sodium phosphate buffer (pH 6.1) containing 1 mM EDTA. The adsorbed enzyme was eluted by use of a linear concentration gradient formed by mixing 5 L of the start buffer with 5 L of 50 mM sodium phosphate (pH 6.1) containing 0.2 M NaCl and 1 mM EDTA. The peak of the thioltransferase activity in the effluent was at about 0.1 M NaCl.

Step 4: Chromatography on Sephadex G-75. The pooled effluent from step 3 (about 2 L) was concentrated to about 150 mL by use of a Millipore Pellicon cassette. It was then applied to a column (9 × 95 cm) of Sephadex G-75 equilibrated with 10 mM sodium phosphate buffer (pH 6.7) containing 1 mM EDTA. The thioltransferase appeared in the effluent after the bulk of the protein components of the sample applied (4.5 L) (cf. Mannervik & Axelsson, 1975).

Step 5: Chromatography on CM-Cellulose II. The pooled effluent from step 4 was applied to a column (1.5 × 12 cm) of CM-cellulose and chromatographed as in step 3. The total volume of the gradient was 200 mL.

TABLE I: Purification of Thioltransferase from Rat Liver.

| step | vol (mL) | total protein (mg) | total act. (units) | spec act. (units/mg) | purification factor (-fold) |
|---|---|---|---|---|---|
| 1. liver supernatant | 1950 | 54 200 | 878 | 0.016 | (1) |
| 2. DEAE-Sephadex | 2310 | 36 300 | 661 | 0.018 | 1.1 |
| 3. CM-cellulose I | 2030 | 6 900 | 463 | 0.067 | 4.2 |
| 4. Sephadex G-75 | 800 | 40 | 178 | 4.45 | 278 |
| 5. CM-cellulose II | 7.5 | 2.18 | 91.6 | 42.0 | 2 630 |
| 6. hydroxylapatite | 19 | 0.8 | 50.1 | 62.6 | 3 910 |
| 7. CM-Sepharose | 1.0 | 0.065 | 45.8 | 705 | 44 100 |

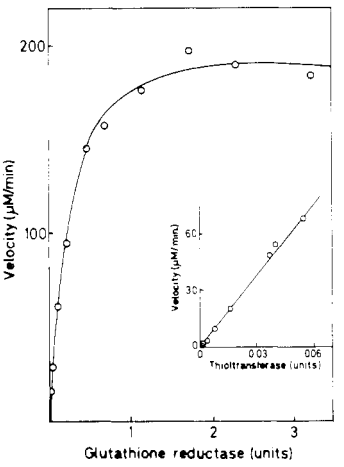


FIGURE 1: Dependence of the thioltransferase assay on the concentration of coupling enzyme (glutathione reductase). Velocity (spontaneous plus thioltransferase-catalyzed) as measured by oxidation of NADPH plotted vs. glutathione reductase concentration. The conditions were as described in the Methods section for the standard assay system using *S*-sulfocysteine as donor substrate. The concentration of thioltransferase used was 0.2 unit/mL in the system. The inset shows the proportionality between velocity and thioltransferase concentration in the standard assay system using a constant concentration (2 units/mL) of glutathione reductase.

Step 6: Chromatography on Hydroxylapatite. The pooled effluent from step 5 was diluted with an equal volume of water and adjusted to pH 6.7 before application to a column (2 × 4 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.7) containing 1 mM EDTA. The thioltransferase was not adsorbed and was eluted with the start buffer.

Step 7: Chromatography on CM-Sepharose. The pooled effluent from step 6 was applied to a column (0.5 × 15 cm) of CM-Sepharose equilibrated with 10 mM sodium phosphate buffer (pH 6.1) containing 1 mM EDTA. After washing the column with the start buffer, the thioltransferase was subjected to a stepwise elution by application of 50 mM sodium phosphate buffer (pH 6.1) containing 0.2 M NaCl and 1 mM EDTA.

## Results

*Assay System.* The thioltransferase-catalyzed reactions were assayed by coupling to the glutathione-reductase-catalyzed reduction of glutathione disulfide (GSSG). The most straightforward analyses are those in which the initial reaction involves a glutathione sulfenyl derivative (RSSG), such as the mixed disulfide of L-cysteine and GSH (CySSG) or *S*-sulfoglutathione ($GSSO_3^-$). In these cases only two separate reactions have to be considered:

$$RSSG + GSH \rightleftharpoons RSH + GSSG$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The first reaction is catalyzed by thioltransferase and the second one by glutathione reductase. By using an excess of glutathione reductase the first reaction becomes rate limiting when the system has reached the steady-state velocity, and the steady-state is attained almost instantaneously. When substrates which are not glutathione derivatives are used (for example, symmetrical disulfides, RSSR), three consecutive reactions are involved:

$$RSSR + GSH \rightleftharpoons RSH + RSSG$$

$$RSSG + GSH \rightleftharpoons RSH + GSSG$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

In this case the two first reactions are catalyzed by the same enzyme (i.e., thioltransferase) and a significant time lag (1–2 min) may appear in the progress curve before steady state is established. Such a time lag cannot normally (in contrast to the previous case) be eliminated by increasing the concentration of the coupling enzyme, because it is dependent on the relationship between the rate constants of the first two reactions, and the rates cannot be varied independently since the reactions are catalyzed by the same enzyme. In these cases the steady-state velocity of the complete system is reported in the present investigation; in the simpler case of two linked reactions, the steady-state velocity is equal to the initial velocity in the time scale of ordinary steady-state kinetic assays. Under all conditions the reactions have been carried out with a concentration of glutathione reductase, which makes the recorded velocities (including the spontaneous reactions) independent of the glutathione reductase-catalyzed reaction. Figure 1 shows that 2 units of glutathione reductase per mL in the assay was sufficient, provided that the overall velocity did not exceed 0.2 mM/min (1.2 $\Delta A_{340nm}$/min). The inset in Figure 1 demonstrates that under these conditions the recorded velocity is proportional to the thioltransferase concentration (after subtraction of the spontaneous reaction).

*Purification of Thioltransferase.* Table I summarizes the results of the purification. The first steps were designed for simultaneous preparation of glutathione *S*-transferases (Askelöf et al., 1975) and glutathione reductase (Carlberg & Mannervik, 1975). A remarkable finding (see Mannervik & Axelsson, 1975) was that the thioltransferase was eluted after most of the proteins on a Sephadex G-75 column. The elution volume of the thioltransferase was in some cases even larger than the mobile phase of the column, indicating retardation by interaction with the gel matrix. As a consequence this purification step was very efficient and gave a purification factor of about 70.

In agreement with previous observations (Eriksson et al., 1974a,b; Mannervik et al., 1974; Mannervik & Eriksson, 1974), the first CM-cellulose chromatography gave rise to two largely overlapping peaks of thioltransferase activity in the

effluent. These two components were pooled, and in different steps later in the purification only a single component could be demonstrated.

Thioltransferase was bound at high ionic strength (2 M) to a matrix of Sepharose 4B substituted with GSH via an ε-aminocaproic acid spacer arm and released by addition of 1 mM dithioerythritol but not by increasing the salt concentration. Although the chromatography on this material was not considered suitable for the purification procedure, it nevertheless indicates that thioltransferase is capable of binding GSH covalently via a disulfide bond as suggested previously for the interaction of the enzyme and free GSH (Eriksson et al., 1974b).

The purity of the isolated thioltransferase was investigated using several electrophoretic methods, including conventional disc gel electrophoresis in three different systems, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and analytical isoelectric focusing. Only one protein component was detected in all systems. However, in the gels after disc electrophoresis the protein band was not sharp, but broad and diffuse. This might be related to the high isoelectric point of the thioltransferase (see below).

*Molecular Weight and Subunit Structure.* The molecular weight was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) and was found to be 11 000. That this value represents the molecular weight of the native enzyme was indicated by the finding that molecular weight estimations by gel filtration on poly(ethylene glycol)-treated, controlled-pore glass beads (cf. Hiatt et al., 1971) in which the elution volume of the enzyme corresponded to a molecular weight somewhat below horse heart cytochrome *c*. Ultracentrifugation in a sucrose density gradient demonstrated a sedimentation velocity, which by comparison with marker enzymes also gave approximately the same molecular weight of thioltransferase.

*Properties of the Purified Enzyme.* The isoelectric point was determined to be at pH 9.55 ± 0.20 ($n$ = 8) by isoelectric focusing in gel rods of polyacrylamide at 22 °C. The purified enzyme was found to contain 8.6% (w/w) carbohydrate. The molecular activity (turnover number) in the standard assay system with *S*-sulfocysteine and glutathione as substrates was 7800 μmol/min per μmol of enzyme (130 $s^{-1}$).

*pH–Activity Profile.* The activity of the thioltransferase as a function of pH was determined with *S*-sulfoglutathione and GSH as substrates. Figure 2 shows a distinct optimum at about pH 7.5. Two different concentrations of glutathione reductase were used in the assay system to check that the coupling reaction did not become rate limiting at any pH value. The dependence of the spontaneous reaction was also determined; it indicates that the degree of ionization of the sulfhydryl group of GSH to the nucleophilic mercaptide form governs the rate of the spontaneous reaction. The p$K$ of this ionization has been determined as 9.2 (Jung et al., 1972). The pH–activity profile of the enzymatic reaction does not reflect the ionization of the sulfhydryl group of free GSH.

*Kinetics of the Thioltransferase-Catalyzed Reactions.* A variety of disulfides and thiosulfate esters of direct or indirect biological interest was tested as substrates in the range of 5–500 μM (for oxytocin up to 1.3 mM). The substrate concentrations were kept relatively low because the normal physiological levels are expected to be low. Use of higher concentrations of *S*-sulfocysteine caused an apparent activation resulting in a molecular activity in the standard assay system (see above), which is higher than the $k_{cat}$ obtained in the low-concentration range (Table II). The glutathione concentration was kept at 4 mM, which is in the physiological

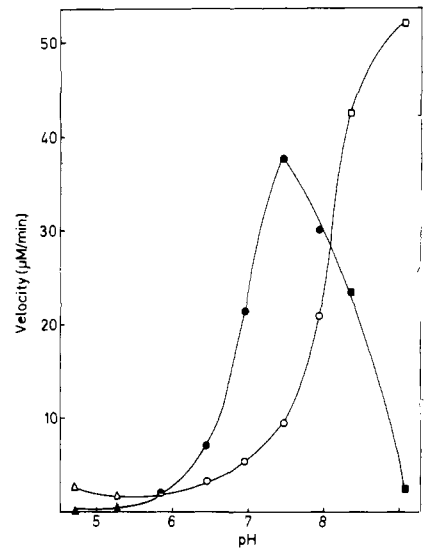


FIGURE 2: Dependence of the reaction between *S*-sulfoglutathione and glutathione on pH. The spontaneous (open symbols) and thioltransferase-catalyzed (filled symbols) reaction velocities were measured as in the standard assay system but *S*-sulfoglutathione (0.137 mM) was substituted for *S*-sulfocysteine. The concentration of thioltransferase was 0.11 unit/mL. The buffers used were 0.1 M Tris-HCl (□), 0.1 M sodium phosphate (○), and 0.1 M sodium acetate–acetic acid (△). The ionic strength was kept constant (0.27 M) by addition of NaCl.

range (Jocelyn, 1972), but somewhat below the level (9 mM) found in rat liver (M. Moron, J. W. DePierre, & B. Mannervik, submitted for publication); the lower value was a compromise made in order to limit the spontaneous reaction and thereby increase the reliability of determinations of the enzymatic reaction. It should also be noted that high concentrations of glutathione result in inhibition of the enzymatic reaction (see below).

Table II shows that three mixed disulfides of glutathione and low-molecular-weight thiols have $K_m$ values of about 50 μM. In the case of the mixed disulfide of coenzyme A, the concentration in liver has been determined as 34 nmol/g wet weight (Dyar & Wilken, 1972), which would correspond to an average concentration of about 50 μM in the cell. The mixed disulfide of L-cysteine and glutathione was also assayed with 1.0 and 10.3 mM GSH. The corresponding $K_m$ values were 20 and 136 μM and the $k_{cat}$ values were $0.34 \times 10^4$ and $1.96 \times 10^4$ $min^{-1}$, respectively. The mixed disulfide of lysozyme has an apparent $K_m$ of 7 μM, but considering that one protein molecule contains 8 half-cysteines, which are all combined with GSH (Axelsson & Mannervik, 1975), the "average $K_m$ value" of a disulfide bond could be considered eightfold higher (56 μM). A fifth glutathione sulfenyl derivative, *S*-sulfoglutathione, has a $K_m$ which is somewhat higher than the above $K_m$ values, possibly due to the negative charge near the scissile S–S bond.

The remaining substrates give kinetic constants, which are less readily interpreted, because they represent values which originate from two consecutive reactions catalyzed by thioltransferase (see Materials and Methods section). The apparent constants nevertheless provide half-saturation concentrations for reduction in the thioltransferase system and are relevant in the consideration of the biological reduction of these substances.

It should be noted that the $k_{cat}/K_m$ ratios for glutathione sulfenyl derivatives (mixed disulfides and *S*-sulfoglutathione), which are naturally occurring, are higher than most of the remaining substrates tested (Table II). The $k_{cat}/K_m$ ratios for the latter substrates are less by a factor ≥10 except for insulin, which gives a value close to those of the glutathione sulfenyl

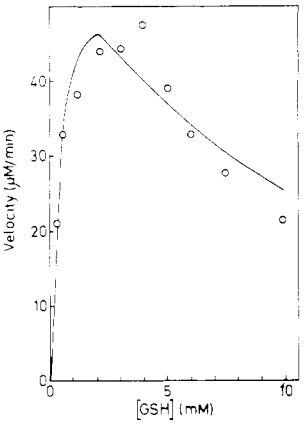


FIGURE 3: Dependence of the thioltransferase-catalyzed reaction on glutathione concentration. The reaction was studied with a constant concentration (0.126 mM) of the second substrate *S*-sulfoglutathione. The curve was obtained by a computer fit of the simple equation for inhibition by excess of substrate (see text). No inhibition by high glutathione concentrations was seen in the spontaneous reaction.

derivatives. It is also apparent that, whereas the velocity of the spontaneous reaction between glutathione and the different substrates varies more than 100-fold, as expressed by the apparent second-order rate constants (Table II), the velocity ($k_{cat}$) of the thioltransferase-catalyzed reactions is much less sensitive to variations in the structure of the donor substrate. Further, low values of the second-order rate constants are reflected in high $K_m$ values. These findings indicate that, as soon as the substrate is bound to the active site of the thioltransferase, much of the steric hindrance and other effects of groups adjacent to the scissile S–S bond are eliminated or neutralized.

In contrast to the disulfides and thiosulfate esters, which gave Michaelian kinetics under the conditions investigated, glutathione concentrations above about 4 mM resulted in inhibition. The concentration of glutathione was varied at a fixed concentration of *S*-sulfoglutathione (Figure 3) or of the mixed disulfide of L-cysteine and glutathione. The following equation was fitted by nonlinear regression to the experimental data:

$$v = \frac{V[\mathrm{GSH}]}{K_m + [\mathrm{GSH}] + K'[\mathrm{GSH}]^2}$$

where $K'$ is a constant corresponding to the inverse of the classical constant for inhibition by excess of substrate. The three constants of the rate equation are evidently functions of the nonvaried substrate concentration, because the reaction involves two substrates. At 0.126 mM *S*-sulfoglutathione concentration the following parameter values were obtained: $K_m = 1.03 \pm 0.44$ mM and $K' = 0.29 \pm 0.13$ mM$^{-1}$.

The fit was not perfect, because the residuals were correlated with the glutathione concentration (Bartfai & Mannervik, 1972; Mannervik & Bartfai, 1973), but the available experimental data were too limited for consideration of more complex rate equations.

## Discussion

The biological function of the thioltransferase studied in the present investigation is probably the reduction of disulfides and thiosulfate esters. Only three reductases catalyzing the cleavage of sulfur–sulfur bonds have been clearly identified: lipoamide dehydrogenase, glutathione reductase, and thioredoxin reductase (Williams, 1976). Each of these enzymes is very specific for its natural disulfide substrate and none of them can serve directly in a more general reductive function. It has been claimed in several reports that additional reductases specific for cystine, *S*-sulfoglutathione, and the mixed disulfide of coenzyme A and glutathione exist (see Mannervik & Eriksson, 1974), but in no case has compelling evidence been presented. The mixed disulfide of coenzyme A and glutathione is a relatively good substrate for glutathione reductase (Carlberg & Mannervik, 1975, 1977), but the thioltransferase-mediated reduction has been estimated to be at least one order of magnitude greater in rat liver (Eriksson et al., 1974c). Other mixed disulfides of glutathione tested are poor substrates for glutathione reductase (Chang & Wilken, 1966; Mannervik & Nise, 1969; Carlberg & Mannervik, 1975); this statement is also applicable to a well-defined mixed disulfide of a protein (lysozyme) and glutathione (Axelsson & Mannervik, 1975). Similarly, *S*-sulfoglutathione is a very poor substrate for glutathione reductase (Winell & Mannervik, 1969). Lipoamide dehydrogenase and thioredoxin reductase are also highly specific for their respective disulfide substrates, lipoamide and thioredoxin (Williams, 1976).

Thus, the available evidence indicates that in vivo most reductions of sulfur–sulfur bonds in disulfides and thiosulfate esters are not catalyzed directly by pyridine nucleotide dependent reductases (Mannervik & Eriksson, 1974), but that

TABLE II: Kinetic Constants for Thioltransferase-Catalyzed and Spontaneous Reactions between Glutathione and Disulfides and Thiosulfate Esters.[a]

| reactant | apparent parameters of enzyme reaction $K_m$ (μM) | $10^{-4}k_{cat}$ (min$^{-1}$) | apparent 2nd-order rate constant of spontaneous reaction, $k$ (mM$^{-1}$ min$^{-1}$) |
|---|---|---|---|
| mixed disulfide of GSH and coenzyme A | 41 | 1.20 | 1.14 |
| L-cysteine | 50 | 0.98 | 0.22 |
| cysteamine | 47 | 0.73 | 0.86 |
| gg-white lysozyme | 7 | 0.18 | 0.44 |
| *S*-sulfoglutathione | 77 | 0.85 | 0.96 |
| *S*-sulfocysteine | 375 | 0.31 | 0.025 |
| L-cystine | 238 | 0.62 | 0.049 |
| L-homocystine | 325 | 0.21 | 0.012 |
| cystamine | 240 | 0.59 | 0.11 |
| *O*-benzoylthiamine propyl disulfide | 515 | 0.89 | 0.025 |
| trypsin | 307 | 0.36 | 0.025 |
| ribonuclease | 302 | 0.19 | 0.007 |
| oxytocin | 1725 | 0.33 | 0.003 |
| insulin[b] | 30 | 0.58 | 0.42 |

[a] Glutathione was used at 4.05 mM concentration and thioltransferase at 0.394 unit/mL (50 nM) in the standard assay system (30 °C). The parameters of the enzymatic reactions were estimated from the plot of Eisenthal & Cornish-Bowden (1974). The $k_{cat}$ values were obtained by assuming that the pure homogeneous thioltransferase has a molecular weight of 11 000 and a specific activity of 705 units/mg. After completion of the series of experiments, it was found that the enzyme used had been partially inactivated by aging. Controls indicated that the $k_{cat}$ values listed should be higher by a factor of 1.5–2 for the fully active enzyme, but lack of enzyme and substrates prevented repetition of the entire series of experiments. The apparent rate constants of the spontaneous reactions were obtained by linear regression analysis of steady-state velocities as functions of the concentration of varied substrate. The functions were straight lines and the apparent second-order rate constants were calculated by dividing the slopes with the (constant) glutathione concentration. [b] The buffer of the assay system was changed to 0.14 M Tris-HCl (pH 8.2).

these reactions are mediated by thiol–disulfide interchange with the reduced (thiol) form of the specific substrate of one of the well-characterized reductases.

In mitochondria disulfides capable of penetrating the inner membrane might be reduced by lipoamide dehydrogenase (Eldjarn & Bremer, 1963). In the cytosol reduced thioredoxin may reduce disulfides and the oxidized thioredoxin formed is subsequently reduced by NADPH and thioredoxin reductase (see Holmgren, 1977). The thioltransferase studied in the present investigation is also localized in the cytosol (Eriksson & Mannervik, 1970a). The thioredoxin system was considered to be less important than the glutathione-dependent system in the reduction of the mixed disulfide of coenzyme A and glutathione (Eriksson et al., 1974c) and in the reduction of *S*-sulfoglutathione (Mannervik et al., 1974), but this view needs confirmation by more direct evidence. Nevertheless, the thioltransferase-catalyzed reactions involving glutathione as reductant, coupled to the regeneration of reduced glutathione by NADPH and glutathione reductase, have to be considered as a major pathway in the biological reduction of sulfur–sulfur bonds in the cytosol.

Apart from a general capacity for reducing sulfur–sulfur bonds in the cytosol, the thioltransferase may also have more specific functions. A role in modulation of the biological function of proteins has been suggested in view of the finding that a very large fraction of glutathione in biological tissues is present in the form of mixed disulfides with proteins (Mannervik & Axelsson, 1975). The diurnal fluctuations in concentrations of protein-mixed disulfides, protein sulfhydryl groups, and reduced glutathione (Isaacs & Binkley, 1977a,b) may be indications of a more integrative control function of glutathione, which is dependent on reactions catalyzed by the thioltransferase. Another specific role of the thioltransferase may be in thiol–disulfide interchange reactions involved in the biosynthesis of proteins (see Poulsen & Ziegler, 1977; Ziegler & Poulsen, 1977).

The molecular properties (such as, for example, molecular weight) and the intracellular localization of the thioltransferase studied in the present work distinguish this enzyme from the protein disulfide isomerase and the glutathione-insulin transhydrogenase, which are both microsomal enzymes and have higher molecular weights (see Hawkins & Freedman, 1976; Drazić & Cottrell, 1977; and references cited in these papers). It has been shown that the thioltransferase activity in rat liver cytosol is about fourfold higher than in microsomes when assayed with a low-molecular-weight substrate such as *S*-sulfocysteine, whereas the cytosolic activity is about threefold lower than the microsomal activity using insulin as substrate (S. Eriksson, unpublished experiments). The presence of several distinct enzymes catalyzing thiol–disulfide interchange in the same cell is an indication of the general importance of these interchange reactions in vivo.

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# Hydroxylamine Oxidoreductase from *Nitrosomonas*: Absorption Spectra and Content of Heme and Metal†

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ABSTRACT: Hydroxylamine oxidoreductase of *Nitrosomonas* accounted for 2.6% of the total cell protein. This soluble enzyme was purified 39-fold with a yield of 27% by ammonium sulfate precipitation and isoelectric focusing. The resulting fraction was 99% pure and had a turnover number of $2.3 \times 10^4$ or $9.5 \times 10^3$ mol $min^{-1}$ (mol of enzyme)$^{-1}$ for hydroxylamine oxidized or nitrite produced, respectively. The enzyme also catalyzed the oxidation of pyrogallol. The value of isoelectric pH was 5.3. The absorption spectrum contained maxima at the following wavelengths (nm): oxidized 408, 534; dithionite reduced 418, 463, 524, 553 (558 shoulder). The enzyme was red in color and had no absorbancy at wavelengths greater than 600 nm. At 77 K the $\alpha$ region of the dithionite-reduced spectrum had maxima at 548 and 557 nm. The substrate $NH_2OH$ caused reduction of 35% of the cytochrome with $\alpha$ maxima at 553 and 558 (548 and 557 at 77 K) but did not cause the appearance of the 460-nm maximum. All cytochromes were autoxidizable, but cytochrome P-460 was oxidized much more rapidly than the others. The 460-nm absorbancy was lost in the presence of CO. A small amount (5%) of the *c*-type heme bound CO. All absorbancy in the 500–560-nm region was accounted for by 18 *c*-type hemes/mol. The enzyme contained 20 mol of iron but no other metals. By difference, 2 mol of Fe was attributed to cytochrome P-460. Heme of P-460 is calculated to have an $\epsilon$ value of 76 $mM^{-1}$ $cm^{-1}$ at 460 nm. Cells contained 5.4 $\mu$mol of *c*-type heme/g of protein. All of the P-460 and 40% of the total cellular *c*-type heme was associated with hydroxylamine oxidoreductase.

Hydroxylamine oxidoreductase from the ammonia-oxidizing autotrophic bacterium, *Nitrosomonas europaea,* catalyzes the aerobic oxidation of hydroxylamine to nitrite in the presence of phenazine methosulfate (PMS).[1] The reaction involves the initial removal of two electrons from hydroxylamine and the subsequent net addition of an atom of oxygen to form nitrite. The enzyme has a particle weight of 200 000 (Rees, 1968) and contains cytochromes absorbing in the range 540–570 nm, which are 30% reducible by hydroxylamine (Falcone et al., 1963; Hooper and Nason, 1965; Rees, 1968; Ritchie and Nicholas, 1974). A CO-binding cytochrome P-460 containing a novel and uncharacterized heme (Rees and Nason, 1965; Erickson and Hooper, 1972; Ritchie and Nicholas, 1974) is unique to the ammonia-oxidizing bacteria and present in fractions containing hydroxylamine oxidoreductase. Treatment of the enzyme with $H_2O_2$ results in simultaneous loss of absorbancy due to P-460, substrate-reducibility of cytochromes, and hydroxylamine dehydrogenase activity, suggesting a role of P-460 in the action of the enzyme (Hooper and Terry, 1977). The present paper represents a procedure for purification of the enzyme to homogeneity with high yields and data on the spectral properties, amino acid composition, metal content, content of heme *c* and heme P-460, and the extinction coefficient of heme P-460.

## Experimental Procedures

*Chemical Assays.* Nitrite, nitrate, hydroxylamine, and diethyl dithiocarbamate (DTC) were assayed as described (Hooper et al., 1977). Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard. Oxygen utilization was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instrument, Yellow Springs, Ohio). Carbohydrate was estimated by reaction with phenol and sulfuric acid (Dubois et al., 1956). Using this assay, horseradish peroxidase (Worthington) was determined to contain 17.6% carbohydrate.

*Metal analysis* was carried out on enzyme samples (2.1 mg of protein) which had been dialyzed against two changes of 2 L of phosphate buffer (10 mM, pH 7.5), containing 10 $\mu$M sodium ethylenediaminetetraacetate. Samples were dried at 60 °C and digested with equal volumes of $HNO_3$, $H_2SO_4$, and $HClO_4$ (van de Bogart and Beinert, 1967). The white ash was dissolved in 2 mL of 1.1 N HCl and iron content determined

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[1] Abbreviations used are: PMS, phenazine methosulfate; DTC, diethyl dithiocarbamate; Tris, tris(hydroxymethyl)aminomethane; DCIP, 2,6-dichlorophenolindophenol; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid.

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