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Purification and Characterization of the Flavoenzyme Glutathione Reductase from Rat Liver*

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Glutathione reductase from rat liver has been purified >5000-fold in a yield of 20%. The molecular weights of the enzyme and its subunits were estimated to be 125,000 and 60,000, respectively, indicating that the native enzyme is a dimer. The enzyme molecular contains 2 FAD molecules, which are reducible by NADPH, GSH, or dithioerythritol. The reduced flavin is instantaneously reoxidized by addition of GSSG. The steady state kinetic data are consistent with a branching reaction mechanism previously proposed for glutathione reductase from yeast (MANNERVIK, B. (1973) *Biochem. Biophys. Res. Commun.* **53,** 1151–1158). This mechanism is also favored by the nonlinear inhibition pattern produced by $NADP^+$. However, at low GSSG concentrations the rate equation can be approximated by that of a simple ping pong mechanism. NADH and the mixed disulfide of coenzyme A and GSH were about 10% as active as NADPH and GSSG, respectively, whereas some sulfenyl derivatives related to GSSG were less active as substrates. The pH activity profiles of these substrates differed from that of the NADPH-GSSG substrate pair.

The ubiquitous tripeptide glutathione (GSH), which is the most abundant low molecular weight thiol in almost all cells (1), is involved in a wide range of enzymatic reactions. A major function of GSH is to serve as a reductant in oxidation-reduction processes; a function resulting in the formation of glutathione disulfide (GSSG). It is well established that numerous additional reactions utilize glutathione in the reduced form (2), and the reduction of GSSG is consequently of fundamental importance for the metabolic function of glutathione. The mammalian liver, which is among the most GSH-rich tissues, exhibits high activities of most of the GSH-dependent biochemical reactions. Therefore, it is of special significance to investigate the enzymatic reduction of GSSG in this tissue. A heat-labile system capable of reducing GSSG was discovered in liver by Hopkins and Elliott (3). Mann in 1932 (4) found that the hepatic GSSG reduction was linked to glucose oxidation by what was later identified as NADPH production in the pentose phosphate pathway. The enzyme directly involved in reduction of GSSG, glutathione reductase, was demonstrated in rat liver by Rall and Lehninger (5) after the discovery of this enzyme in plant tissues (6, 7). The present study is warranted by the fact that relatively little is known about hepatic glutathione reductase and that published data on highly purified rat liver enzyme (8, 9) indicate that the molecular properties are significantly different from those of glutathione reductase from other sources.

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EXPERIMENTAL PROCEDURE

Materials

NADPH, $NADP^+$, FMN, FAD, GSH, cystine, coenzyme A, bovine serum albumin, cytochrome *c* (horse heart), and ovalbumin were obtained from Sigma; L-lactate dehydrogenase (rabbit muscle), alcohol dehydrogenase (yeast), L-malate dehydrogenase (pig heart), carbonic anhydrase (bovine erythrocytes), and GSSG from Boehringer; CM-cellulose CM32 and DEAE-cellulose DE32 from Whatman; Sephadex G-25, G-75, G-100, and G-150 from Pharmacia; hydroxylapatite from Bio-Rad Laboratories.

CoASSG[1] (10), CySSG (11), and $GSSO_3H$ (12) were synthesized according to procedures previously described by one of the authors (B.M., formerly Bengt Eriksson) and his co-workers. The purity of these compounds was checked by paper electrophoresis.

Assay of Glutathione Reductase Activity

The rate of oxidation of NADPH by GSSG at 30° was used as a standard measure of enzymatic activity (13). The reaction system of 1 ml contained: 1.0 mM GSSG, 0.1 mM NADPH, 0.5 mM EDTA, 0.10 M sodium phosphate buffer (pH 7.6), and a suitable amount of the glutathione reductase sample to give a change in absorbance of 0.05 to 0.30/min. The oxidation of 1 μmol of NADPH/min under these conditions is used as a unit of glutathione reductase activity. The specific activity is expressed as units per mg of protein.

Determination of Protein Concentration

Protein concentration was calculated from the absorbance at 260 and 280 nm (14). In the purest enzyme fractions the estimations were based on the value of 1.86 (15) for the absorbance (per cm) at 280 nm for a solution containing 1 mg of protein/ml.

[1] The abbreviations used are: CoASSG and CySSG, the mixed disulfides of coenzyme A and glutathione and of cysteine and glutathione, respectively; CySSCy, cystine; $GSSO_3H$, *S*-sulfoglutathione.

Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed essentially according to Ornstein (16) and Davis (17). Proteins were stained by Coomassie brilliant blue G-250.

Sodium dodecyl sulfate gel electrophoresis was carried out according to Weber and Osborn (18).

Estimation of Molecular Weight by Gel Filtration

A column (2 × 80 cm) of Sephadex G-100 or G-150 was loaded with a sample of 2 ml of purified rat liver glutathione reductase, which in addition contained (molecular weight): L-lactate dehydrogenase (140,000), alcohol dehydrogenase (150,000), L-malate dehydrogenase (70,000), carbonic anhydrase (30,000), and cytochrome *c* (12,400). The eluent was 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl. The elution volumes of the components were determined by standard enzymatic assay methods. Carbonic anhydrase activity was assayed with *p*-nitrophenyl acetate; the identity of the esterase activity was established by its sensitivity to inhibition by acetazolamide (19).

Kinetic Analysis

The investigation of the steady state kinetics of the purified glutathione reductase (specific activity >200 units/mg) was carried out on an Aminco-Chance DW-2 dual wavelength spectrophotometer. Fitting of rate equations to experimental data was carried out by a Gauss-Newton nonlinear regression program as previously described (20).

Purification of Glutathione Reductase

Step 1: Preparation of Liver Supernatant—Livers (about 400 g) were obtained from 40 male Sprague-Dawley rats (200 to 250 g), which were killed by decapitation. The livers were cut into small pieces and homogenized in 9 ml of 0.25 M ice-cold sucrose per g of rat liver in a Turmix blender. All subsequent manipulations were carried out at about 4°. The homogenate was centrifuged for 45 min at 14,000 rpm. The pellets were suspended in a small volume of 0.25 M sucrose and centrifuged. The supernatants were combined with the previous centrifugate. The pooled material was adjusted to pH 5.5 with cold 0.2 M acetic acid and centrifuged as described above (Fraction 1).

Step 2: Gel Filtration on Sephadex G-25—The centrifugate was pumped through a Sephadex G-25 coarse column (30 × 35 cm) equilibrated with 10 mM sodium phosphate, pH 6.1. From this stage on all buffer solutions were 1 mM with respect to ethylenediaminetetraacetate. The protein-containing effluent was collected (Fraction 2).

Step 3: CM-cellulose Chromatography I—The gel-filtered material was applied to a CM-cellulose column (height 5 cm, diameter 9 cm) equilibrated with the buffer used in the previous step. When the total volume had been introduced, the bed was rinsed with buffer to elute unadsorbed protein. The enzyme was eluted by application of a gradient formed by linear mixing of 2000 ml of 10 mM sodium phosphate, pH 6.1, and 2000 ml of 50 mM sodium phosphate, pH 6.1, containing 0.2 M NaCl. The effluent containing glutathione reductase and glutathione *S*-aryltransferase was saved (Fraction 3).

Step 4: Gel Filtration on Sephadex G-75—The pooled material from Step 3 was concentrated to 29 ml by ultrafiltration on a Diaflo PM10 membrane before gel filtration on a Sephadex G-75 column (4 × 110 cm), equilibrated with 10 mM sodium phosphate, pH 6.7, Glutathione reductase was eluted before glutathione *S*-aryltransferase and was pooled separately (Fraction 4).

Step 5: DEAE-cellulose Chromatography I—Fraction 4 was adjusted to pH 8.5 by repeated concentration on a Diaflo PM10 membrane and dilution with the buffer used in the subsequent chromatography on DEAE-cellulose. The final sample volume was 69 ml. The bed (4 × 5 cm) was equilibrated with 20 mM Tris-HCl, pH 8.5, and elution was effected by a linear gradient of KCl 0 to 0.3 M (total volume, 1000 ml), formed in the start buffer (Fraction 5).

Step 6: Hydroxylapatite Chromatography—Fraction 5 was equilibrated with 10 mM potassium phosphate, pH 6.7, by ultrafiltration as described in Step 5 and concentrated to 28 ml before application on a hydroxylapatite column (3 × 5 cm) packed in the same buffer. Elution was effected by a linear ionic strength gradient of potassium phosphate, 10 to 500 mM, pH 6.7 (total volume, 400 ml) (Fraction 6).

Step 7: CM-cellulose Chromatography II—The enzyme was dialyzed against 10 mM sodium phosphate, pH 6.1, and chromatographed on a CM-cellulose bed (1 × 7 cm) as in Step 3. The total volume of the gradient was 100 ml (Fraction 7).

Step 8: DEAE-cellulose Chromatography II—Before this step, 5 ml of Fraction 7 were passed through a Sephadex G-25 Fine column (2 × 11 cm) equilibrated with 20 mM Tris-HCl pH 8.2. The enzyme then was adsorbed on a DEAE-cellulose column (1 × 5 cm) packed in the same buffer. Elution was effected by a linear KCl gradient, 0 to 0.3 M (total volume, 20 ml), in the buffer. The fraction containing the highest activity is denoted Fraction 8. This fraction, which was slightly yellow but not fluorescent, was used in the characterization of the enzyme.

RESULTS AND DISCUSSION

Purification of Glutathione Reductase—Table I summarizes the results of the purification. The enzyme can be purified reproducibly to a specific activity of >200 units/mg in a yield of about 20%. A slight improvement of the specific activity can be obtained by a more restrictive pooling of fractions of the first CM-cellulose chromatography because the first purification steps were designed for the simultaneous preparation of glutathione *S*-aryltransferase (21).

Working with smaller amounts of enzyme, we sometimes have obtained a higher specific activity (about 300 units/mg) than that given in Table I, but in these cases the low enzyme concentrations introduced uncertainty in the protein determinations. All reliable figures of the specific activity seem to approach a limiting value of ≤250 units/mg. Extension of the purification procedure did not increase significantly the purity of the enzyme.

Attempts also have been made to purify glutathione reductase by affinity chromatography on Sepharose 4B containing glutathione derivatives or $NADP^+$ as ligands. However, this method has not so far been included in the purification scheme. Neither has this technique given a higher specific activity of glutathione reductase than about 250 units/mg.

Analysis by disc electrophoresis of the purest fraction of Table I demonstrated only trace amounts of one or maybe two protein bands in addition to the major component. This finding and the flavin analysis (see below) indicate that the enzyme is close to the state of homogeneity.

Comparison of the present results with the data of Mize and Langdon (8) on rat liver glutathione reductase shows a significant difference in specific activity between the two preparations. Mize and Langdon (8) give a value of 839 units/mg, which is about 4-fold higher than our seemingly almost homogeneous enzyme. Minor differences in the assay systems are insufficient to provide an explanation for the discrepancy. However, their data show that the total amount of protein in their purest fraction was in the microgram range, which might be difficult to estimate accurately.

Highly purified mammalian glutathione reductase has been obtained previously also from human erythrocytes (22, 23) and the specific activities of the preparations were 145 and 165

TABLE I

Purification scheme of glutathione reductase from rat liver

Fraction	Volume	Total protein	Total activity	Specific activity
	ml	*mg*	*units*	*units/mg*
1	4050	34100	1260	0.037
2	8300	21900	1330	0.061
3	540	1800	1030	0.57
4	140	439	921	2.10
5	95	46	593	12.8
6	27	6.9	453	65.7
7	5.6	6.5	490	75.3
8	3.25	1.2	249	207

units/mg. We are not, with the exception of the previous report on glutathione reductase from rat liver (8), aware of any preparation of the mammalian enzyme, which has a higher specific activity than that obtained in the present investigation. The corresponding values of the enzyme purified from yeast (15), *Penicillium chrysogenum* (24), *Escherichia coli* (25), or rice embryos (26) range from 150 (rice) to 525 (*E. coli*).

Molecular Weight and Subunit Structure—The molecular weight of glutathione reductase from rat liver was estimated to about 125,000 (Fig. 1). This molecular weight is consistent with a molecular weight of about 60,000 for the subunits of glutathione reductase, determined by sodium dodecyl sulfate gel electrophoresis (Fig. 2), if the enzyme is assumed to contain two subunits. A dimeric structure and a molecular weight in the range of 105,000 to 130,000 have previously been established for glutathione reductase from yeast (27) and rice embryos (26). The finding of 2 mol of flavin/mol of enzyme (see below) is consistent with such a subunit structure of our rat liver enzyme as are the data on the enzyme from yeast (15, 27), rice embryos (26), erythrocytes (22, 23), and *Penicillium* (24).

Our estimate of the molecular weight differs considerably from the previously published value of 44,000 (9) and the recent report of 42,500 (28) for the rat liver enzyme. On the other hand, our data are consistent with all other determinations on homogeneous preparations from different sources. The possibility was considered that the discrepancy was due to dissociation of the enzyme into monomers under some conditions. However, we have performed molecular weight estimations by gel filtration under a variety of conditions including high and low enzyme concentrations and high and low ionic strength, but never obtained any estimates significantly lower than 125,000. Furthermore, it should be noted that the flavin analysis and sodium dodecyl sulfate electrophoresis independently give a value ≥60,000 for the molecular weight of the monomer of our preparation. It does not seem possible to resolve this controversy without direct comparison of the different preparations.

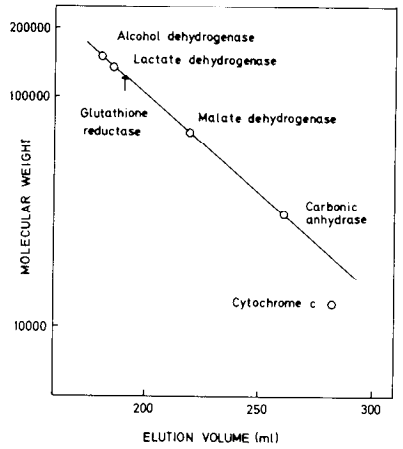

FIG. 1. Estimation of the molecular weight of glutathione reductase by gel filtration on Sephadex G-150. The experiment was carried out as described in the text.

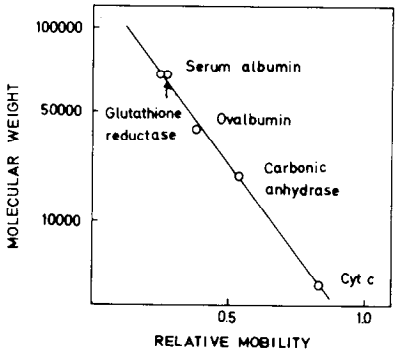

FIG. 2. Estimation of the molecular weight of the subunits of glutathione reductase by sodium dodecyl sulfate gel electrophoresis. The experimental procedure of Weber and Osborn (18) was followed. *Cyt c*, cytochrome *c*.

Flavin Content—The purified glutathione reductase had a visible absorption spectrum (Fig. 3) typical of flavoproteins, which was very similar to spectra previously published for the enzyme from other sources (15, 22–24, 26). The spectrum had peaks at 379 and 463 nm, minima at 325 and 407 nm, and a shoulder at 485 nm. The absorbance ratios A_{280}:A_{463} and A_{379}:A_{463} were 8.2 and 0.98, respectively, in good agreement with published spectral data. The flavin was released from the protein by boiling of the enzyme for 3 min and identified with FAD by comparison of its electrophoretic migration with that of reference substances (FMN and FAD) (29). Further evidence for the identification of the prosthetic group was obtained by the finding that the inactive apoenzyme, prepared by acid ammonium sulfate precipitation, was reactivated by FAD but not by FMN (Table II).

Langdon and co-workers were unable to identify flavin in their enzyme preparation (9), but Buzard and Kopko (30) have shown that crude rat liver glutathione reductase is inactivated by acid ammonium sulfate treatment and reactivated to some extent by addition of FAD.

TABLE II

Preparation of inactive apoenzyme and restoration of activity

Purified glutathione reductase (7 ml) was mixed at room temperature (22°) with an equal volume of saturated ammonium sulfate and acidified by 2.45 ml of 0.1 M HCl. The precipitate formed was collected by centrifugation and dissolved in 7 ml of 0.1 M sodium phosphate, pH 7.4, containing 1 mM EDTA. The apoenzyme thus prepared was divided into 0.5-ml portions, some of which were supplemented with flavin cofactors. The different aliquots were incubated for 30 min at 30° before determination of the enzymatic activity. The concentrations of flavin indicated refer to the conditions in the enzyme stock solutions.

Enzyme	Activity
Untreated control	100
Apoenzyme	1.9
Apoenzyme + FMN (5 μM)	1.9
Apoenzyme + FMN (20 μM)	1.6
Apoenzyme + FAD (5 μM)	34.6

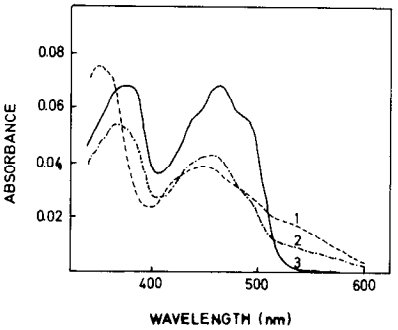

FIG. 3. Absorption spectrum of reduced and spontaneously reoxidized glutathione reductase. The amount of enzyme-bound flavin was 3.0 nmol, and the original spectrum was identical to *Spectrum 3*. Reduction was effected by addition of 10 nmol of NADPH, after which *Spectrum 1* was immediately recorded. Recording of *Spectra 2* and *3* were started 5 and 10 min, respectively, after recording of *Spectrum 1*.

Based on the extinction coefficients of yeast glutathione reductase at 280 and 460 nm (15), the FAD content of our preparation was 2.4 mol/125,000 g of enzyme. In a different preparation of rat liver glutathione reductase, a molar ratio of 2.2 was calculated on the same basis. Thus, the enzyme molecule appears to contain 2 molecules of FAD.

Reduction and Reoxidation of the Enzyme-bound Flavin—The flavin chromophore was reduced by addition of a stoichiometric amount of NADPH, but was rapidly reoxidized as the reaction was carried out aerobically (Fig. 3); similar results were first obtained by Colman and Black with the yeast enzyme (31). Excess of NADPH increased the lifetime of the reduced form. The rat liver enzyme, like the yeast enzyme (15, 31), also could be reduced by GSH or dithioerythritol. Addition of GSSG to the enzyme reduced by NADPH caused an immediate reoxidation of the flavin, which was too rapid to measure. The experimental conditions were identical with those of Fig. 3 except for the addition of a 5-fold molar excess of GSSG before recording of the spectrum. After GSSG addition no sign of the reduced form of the flavin could be detected. This finding is consistent with the assumption that the flavin prosthetic group is functional in the catalytic mechanism of the enzyme.

Steady State Kinetics—Initial velocities were plotted in v *versus* v/[NADPH] (Fig. 4) or v *versus* v/[GSSG] diagrams, resulting in lines which at low concentrations of the constant substrate seemed to intersect in one point on the x axis. This intersecting pattern is typical for a ping pong mechanism (32) and has previously been observed for the enzyme from other sources. Fitting the rate equation

$$v = \frac{V[\text{GSSG}][\text{NADPH}]}{K_m^G[\text{NADPH}] + K_m^N[\text{GSSG}] + [\text{GSSG}][\text{NADPH}]}$$

where V is the maximal velocity, and K_m^G and K_m^N are Michaelis constants for GSSG and NADPH, respectively, to the experimental data in which the GSSG concentrations was

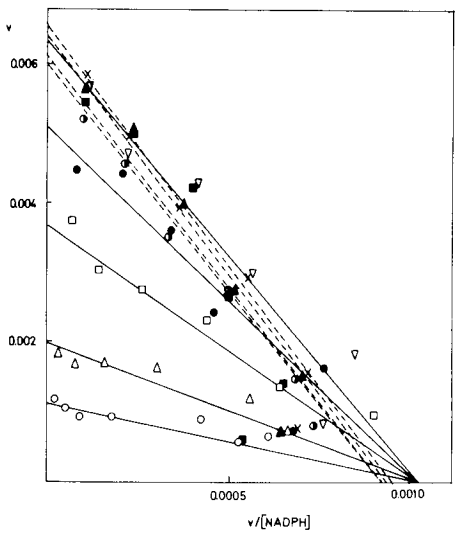

FIG. 4. Effect on initial velocity of variation of the NADPH concentration at different constant concentrations of GSSG: ○, 0.0088; △, 0.0176; □, 0.044; ●, 0.088; ▲, 0.179; ■, 0.44; ×, 0.88; ▽, 2.64; ◑, 5.28 mM. The enzyme concentration was 0.3 nM. The *solid lines* were calculated on the basis of the rate law of a simple ping pong mechanism for the GSSG concentrations ≤ 179 μM. The *dashed lines* were calculated for the highest GSSG levels on the basis of fitting the rate equation of a branching mechanism (33, 34) to the complete data set. Velocities and concentrations are expressed in micromoles per min and millimolar, respectively.

≤179 μM gave the following estimates of the constants: V = 14,400 ± 500 μmol/min/μmol of flavin; K_m^G = 56.7 ± 0.4 μM; and K_m^N = 7.9 ± 0.6 μM. The values obtained for V and K_m^G are remarkably similar to the corresponding values reported by Massey and Williams for the yeast enzyme (15,000 and 55 μM, respectively) (15). The flavin content of the rat liver enzyme was calculated on the basis of an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 463 nm (see Ref. 15). However, the v *versus* v/[GSSG] plot was not linear at high concentrations of GSSG and demonstrated inhibition by excess of substrate. As a result of this feature the lines corresponding to the highest constant GSSG concentrations (*dashed*) appear below lines of intermediately high concentrations in Fig. 4. Consequently, a more complex rate law than that of a simple ping pong mechanism is required to account for the complete data set. The rate equation

$$v = \frac{V_1[\text{GSSG}][\text{NADPH}] + V_2[\text{GSSG}]^2[\text{NADPH}]}{K_1[\text{GSSG}] + K_2[\text{NADPH}] + [\text{GSSG}][\text{NADPH}] + K_3[\text{GSSG}]^2 + K_4[\text{GSSG}]^2[\text{NADPH}]}$$

previously proposed for the kinetics of yeast glutathione reductase (33, 34) actually proved to be a good model for description of the experimental data; the fit was significantly better than that provided by the equation for a ping pong mechanism as demonstrated by comparison of the residual sums of squares and residual plots after regression analysis according to the two models. The estimates of the constants are given in Table III; V_1, K_1, and K_2 correspond to V, K_m^N, and K_m^G, respectively, in the simpler rate equation.

$NADP^+$ was found to be a noncompetitive inhibitor with respect to NADPH. The inhibition was 50% with 100 μM $NADP^+$ when the concentrations of NADPH and GSSG were 10 μM and 1.0 mM, respectively. A nonlinear inhibition pattern was observed when the $NADP^+$ concentration was varied at constant substrate concentrations (Fig. 5). This inhibition pattern contradicts a simple ping pong mechanism and gives further support to the proposition that the branching mechanism previously advanced for yeast glutathione reductase (33, 34) may apply also for the rat liver enzyme. In fact, the inhibition by $NADP^+$ predicted on the basis of the branching mechanism is a 2:1 function (see Ref. 35 for nomenclature), which is consistent with the appearance of Fig. 5.

Substrate Specificity and pH Dependence—The purified enzyme was capable of using NADH as substrate instead of NADPH. The activity obtained with 0.1 mM NADH was under optimal conditions about 10% of that obtained with NADPH. However, the pH dependence of the two activities were significantly different (Fig. 6). Optimal activities were obtained at pH 5.0 to 5.5 and pH about 7.0 for NADH and

TABLE III

Estimated kinetic constants of rate equation of branching mechanism

The data are presented in Fig. 4. Units of the constants are expressed in their appropriate dimensions by using micromoles per min per μmol of flavin as the basic unit of velocity and by using micromolar concentration.

Constant	Value (± S.E.)
V_1	14,800 ± 1,000
V_2	39.3 ± 21.1
K_1	8.1 ± 1.0
K_2	62.9 ± 8.2
K_3	0.022 ± 0.012
K_4	0.0039 ± 0.0020

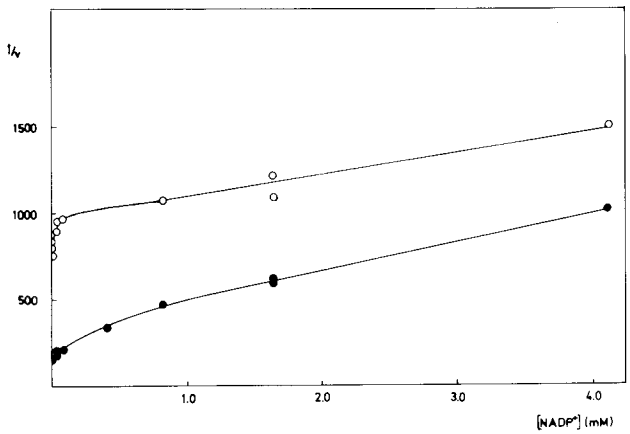

FIG. 5. Effect of $NADP^+$ on the reciprocal initial velocity at two different GSSG concentrations: ○, 2 μM; ●, 10 μM. The NADPH concentration was 10.7 μM. The standard assay conditions were used. Velocities and concentrations are expressed in micromoles per min and millimolar, respectively.

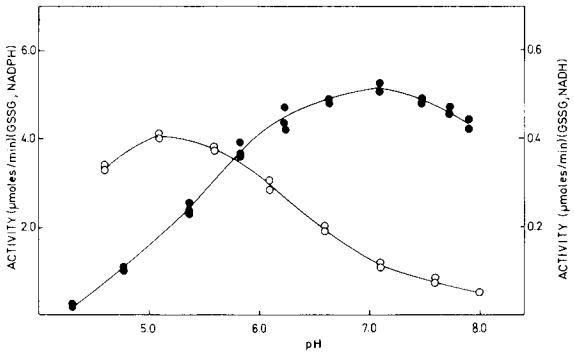

FIG. 6. pH dependence of initial velocities obtained with NADPH and NADH. The pyridine nucleotides were used at 0.1 mM concentration: ●, NADPH; ○, NADH. The GSSG concentration was 0.5 mM. Sodium phosphate (50 mM) was included in the entire pH range investigated. At about pH 6 and below, sodium acetate (50 mM) was included as a buffering substance. The pH values were measured with a pH meter in the reaction mixtures. The spontaneous decrease of absorbance of NADPH and NADH at acid pH values was subtracted from the velocities.

NADPH, respectively. Different pH optima for the pyridine nucleotides were first noted for the erythrocyte enzyme (36), with which the activity with NADH is about 20% of that determined with NADPH under standard assay conditions (22, 36). The pH optimum previously determined for the rat liver enzyme covered a range of pH 7 to 8.5 (8) in fair agreement with the present data. NADH, on the other hand, was reported to be <1% as active as NADPH for the same enzyme preparation (8).

Table IV summarizes experiments on the activity of the purified rat liver glutathione reductase with naturally occurring disulfides and with *S*-sulfoglutathione ($GSSO_3H$). Two concentrations of the substrates were used to evaluate the degree of saturation of the enzyme with the different substrates. The mixed disulfide of coenzyme A and GSH (CoASSG) was next to GSSG the best substrate. The activity of rat liver glutathione reductase with $GSSO_3H$ has previously been found to be about 0.5% of the activity with GSSG (37). Cystine and CySSG are also very poor substrates. The finding that the highly purified rat liver glutathione reductase indeed catalyzes the NADPH-linked reduction of CoASSG provides further support to the previous conclusion that no separate reductase exists in rat liver

TABLE IV

Activity of purified rat liver glutathione reductase with naturally occurring disulfides and S-sulfoglutathione

Assays were carried out in the standard assay system in which the substrates indicated were substituted (in two different concentrations) for GSSG. The activity values given correspond to 0.30 μM enzyme-bound flavin.

Substrate	Activity	
	0.5 mM	2.0 mM
	μmol/min	
GSSG	2.84	2.98
CoASSG	0.21	0.40
CySSG	0.050	0.165
$GSSO_3H$	0.002	0.012
CySSCy	<0.001	<0.001

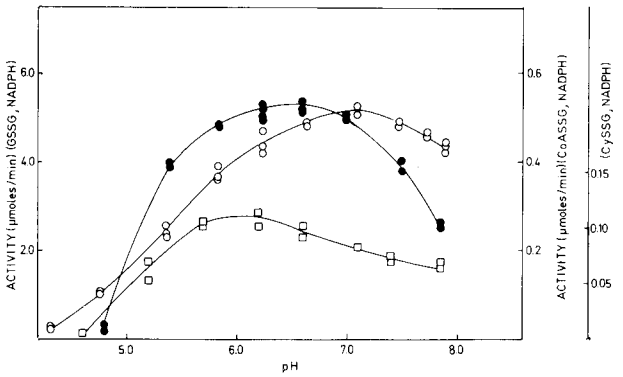

FIG. 7. pH dependence of initial velocities obtained with different disulfide substrates. The disulfides were used at 0.5 mM concentration: ○, GSSG; ●, CoASSG; □, CySSG. The NADPH concentration was 0.1 mM. The buffering ions were the same as in Fig. 6. The spontaneous decrease of absorbance of NADPH at acid pH values was subtracted from the velocities.

for this substrate (38). The biological reduction of low molecular weight disulfides and thiosulfate esters in the cytosol is considered to involve GSH and a thioltransferase as additional catalysts (39, 40).

The pH dependence of the CoASSG and CySSG reductions were found to have optima at lower pH values than the reduction of GSSG (Fig. 7). The increase of the activity obtained with NADH and CoASSG at low pH values demonstrates that glutathione reductase is less specific in this pH range. A possible explanation for this effect is that the structure of the enzyme is less rigid at a lower pH value, causing a greater latitude in the binding to the active center of different molecules of both donor and acceptor substrates. Whether the decreased specificity at lower pH values has any physiological implications cannot be decided presently.

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