BBA 65769

ISOLATION AND CHARACTERIZATION OF GLUTATHIONE REDUCTASE FROM *PENICILLIUM CHRYSOGENUM**

TERRY S. WOODIN AND IRWIN H. SEGEL

Department of Biochemistry and Biophysics, University of California, Davis, Calif. (U.S.A.)

(Received February 29th, 1968)

SUMMARY

Glutathione reductase (NADPH:glutathione oxidoreductase (EC 1.6.4.2)) was purified 3000-fold from *Penicillium chrysogenum*. The purified preparation is homogeneous according to: (a) its behavior on disc gel electrophoresis, (b) sedimentation in the analytical ultracentrifuge and (c) elution from a Sephadex G-200 column. The enzyme has a pH optimum between 6.8 and 7.6, and its activity is affected by ionic strength (optimum in 0.1 M phosphate). The enzyme is inhibited by *p*-chloromercuribenzoate (PCMB), *N*-ethylmaleimide and heavy metals. Kinetic parameters of the enzyme are: (a) a mol. activity of 46 000 moles of NADPH oxidized/min per mole of enzyme, (b) a $K_{m\,\mathrm{NADPH}}$ of 10^{-5} M, a $K_{m\,\mathrm{GSSG}}$ of $5.5 \cdot 10^{-5}$ M and a $K_{m\,\mathrm{NADH}}$ of $3 \cdot 10^{-4}$ M. The enzyme has absorption maxima at 462, 365 and 275 mμ, which is typical of a flavin enzyme. The flavin is FAD. Other physical parameters of the enzyme are: (a) an $E^{1\%}_{1\,\mathrm{cm}}$ at 280 mμ of 18.6, (b) a molecular weight of 110 000, with 2 flavins/mole of enzyme and (c) a diffusion coefficient of $5.15 \cdot 10^{-7}$ cm²/sec.

INTRODUCTION

Glutathione reductase (NADPH:glutathione oxidoreductase (EC 1.6.4.2)) is widely distributed in nature. The enzyme has been demonstrated in plant extracts[1-4], various animal tissues[5-12], bacteria[13-15], insects[16], yeast[17,18] and *Neurospora crassa*[19], and extensively purified from *Escherichia coli*[13], pea seedlings[4], rat liver[20], human erythrocytes[21] and yeast[17,18] but not from *Penicillium chrysogenum* or any other filamentous fungus.

The experiments described in this paper were designed to isolate and characterize the glutathione reductase from *P. chrysogenum*. Our interest in this enzyme was stimulated in part by experiments described elsewhere[22] that suggest a role for glutathione reductase in the metabolism of cysteine-*S*-sulfate.

Abbreviations: PCMB, *p*-chloromercuribenzoate; GSH, glutathione (reduced); GSSG, glutathione (oxidized).

* Some of the work described in this paper was taken from a thesis submitted to the Graduate School of the University of California by T. S. W. in partial fulfillment of the requirements for the Ph. D. degree in Comparative Biochemistry.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from commercial sources and used without further purification.

Enzyme assay. For routine assays the rate of NADPH oxidation was followed by measuring the decrease in absorption at 340 mμ ($\Delta A_{340\ m\mu}$) on a Cary Recording Spectrophotometer. The assay mixture contained 0.5 μmole EDTA, 5 μmoles glutathione (oxidized) (GSSG) and 0.1 to 0.2 μmole NADPH in a final volume of 1 ml of 0.1 M phosphate buffer (pH 7.4). A unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmole of NADPH in 1 min at 25° under optimum conditions. During purification of the enzyme, 3 different protein assays were employed: (a) the biuret method[23], using bovine serum albumin as a standard (b) absorption at 280 mμ, using an $E_{1\ cm}^{1\ \%}$ at 280 mμ of 18.6 (ref. 17), and (c) the 215–225 mμ absorption method of WADDELL[24].

Growth and harvest of cells. For the large-scale preparation of pure glutathione reductase from *P. chrysogenum*, the organism was grown at 25° on Citrate No. 3 synthetic medium[25] containing 1 g/l Na_2SO_4 as the sulfur source and a few drops of antifoam (Union Carbide Y4988 Silicone). The culture was grown with vigorous aeration (600 rev./min impeller speed, 15 cubic feet per min aeration) for 2 days in 12-l New Brunswick Microferm Laboratory fermenters. Approx. 500 g (wet pressed weight) of mycelia were obtained from each fermenter.

The cells were harvested by filtering the thick mycelial suspension through a sintered-glass funnel. The mycelial pad was washed 5 times by resuspension in distilled water and refiltering. The washed mycelia were stored frozen.

Extraction of protein from the cells. Frozen mycelia were broken into small pieces and ground for 25 min in a Waring blender (model CB-5, one gallon capacity) with glass beads (Vir-Tis) and 0.1 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA. A ratio of 1 g mycelia, 1.5–2.0 g beads and 1.5–2.0 ml buffer was employed. The temperature was kept below 10° by grinding in the cold room for 3 to 6 min at a time and stopping when the temperature exceeded 10°. The mixture was then cooled to 0° in an ice-salt bath and the grinding continued. All subsequent operations were carried out at 4°.

The mixture was centrifuged at 27 000 × *g* and the supernatant solution saved. The precipitate was washed twice with the buffer using first an equal volume and then a one-half volume of the amount used for the extraction. The supernatant solution and washings were combined and designated "crude extract". The specific activity of the crude extracts varied between 0.03–0.3 μmole NADPH oxidized/mg protein per min. This variation in specific activity seemed related to the efficiency of protein extraction. Those crude preparations having the most protein had the lowest specific activity. All preparations had similar specific activities after the first ammonium sulfate fractionation.

First ammonium sulfate precipitation. The crude extract was treated with solid ammonium sulfate (40% saturation; 242 g/l). The salt was added in 2 large additions. After all the salt crystals had dissolved, the pH of the solution (which usually dropped to about 6.8), was carefully adjusted to 7.4 with 5 M KOH. The mixture was stirred for 4–6 h. The precipitate was then collected by centrifuging for 10 min at 27 000 × *g* and discarded.

The supernatant solution was brought to 60% saturation with ammonium sulfate (an additional 130 g/l) by rapid addition of the salt. Again the pH (which had dropped to about 7.2) was readjusted to 7.4. The solution was stirred for 4–6 h and the precipitate collected by centrifuging for 10 min at 27 000 × *g*.

The protein precipitating between 40–60% ammonium sulfate saturation was dissolved in 0.05 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA and dialyzed against 2 changes of the same buffer (6 l each time).

Acetone precipitation. The dialyzed enzyme preparation was adjusted to contain 15–20 mg protein/ml since lower protein concentrations resulted in incomplete precipitation of glutathione reductase. Aquacide (Cal Biochem, Grade II) was used to concentrate the protein. The enzyme solution was brought to −3° by stirring in an ice-salt bath. Acetone was then slowly dripped in from a separatory funnel. That portion of the protein solution precipitating between 40 and 60% (v/v) acetone was collected, dissolved in 0.01 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA, and dialyzed overnight against a 10-fold excess of the same buffer.

Second ammonium sulfate precipitation. The second precipitation with $(NH_4)_2SO_4$ was similar to the first. The pH of the solution was adjusted after each addition of $(NH_4)_2SO_4$ and the solutions equilibrated 4–6 h before collection of the precipitate. The protein precipitating between 45 and 60% ammonium sulfate saturation was collected, dissolved in 0.01 M phosphate (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA and dialyzed against two changes of a 10-fold excess of the same buffer.

DEAE fractionation. The dialyzed preparation from above was applied to a DEAE cellulose (Schleicher and Schuell) ion exchange column (5 cm × 36 cm) and eluted with a linear gradient of pH 7.4 phosphate buffer (0.01 to 0.5 M). Those fractions containing glutathione reductase activity were combined, placed into dialysis tubing and concentrated with aquacide to a volume of 10 to 20 ml.

Sephadex G-200 fractionation. The DEAE eluates were divided into 5 ml aliquots. These were applied separately to a Sephadex G-200 (Pharmacia) column (3 cm × 38 cm) equilibrated with 0.1 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA and eluted with the same buffer. Those fractions containing glutathione reductase were combined and dialyzed against 0.01 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA.

TEAE cellulose fractionation. Fractions from the Sephadex column were applied to a TEAE-cellulose (Schleicher and Schuell) ion exchange column and eluted with a linear gradient of phosphate buffer 0.01 to 0.5 M. Those portions exhibiting glutathione reductase activity were combined and dialyzed against 0.01 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA.

Concentration of the protein. The eluates from the TEAE-cellulose column were applied to a small (1 cm × 8 cm) TEAE-cellulose ion exchange column. A sharp yellow band formed at the top of the column which was eluted with 0.1 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA. The protein was usually collected in a volume of 1–5 ml.

The data from a typical purification procedure are given in Table I.

RESULTS AND DISCUSSION

Proof of purity. The purified enzyme exhibited one band on disc gel electro-

TABLE I

PURIFICATION OF *P. chrysogenum* GLUTATHIONE REDUCTASE

Step	*Total vol. (ml)*	*Concn. protein (mg/ml)*	*Specific activity (μmoles NADPH oxidized/ mg protein per min)*	*Total protein (mg)*	*Total units*	*Yield (%)*	*Purification factor*
1. Crude extract	3300	10.6	0.13	35 000	4550	100	1.0
2. 40–60% $(NH_4)_2SO_4$ ppt.	440	22.8	0.45	10 000	4500	100	3.5
3. 40–60% (v/v) acetone ppt.	100	12.0	2.5	1 200	3000	70	20
4. 45–60% $(NH_4)_2SO_4$ ppt.	90	5.5	5.4	500	2700	63	40
5. DEAE-cellulose	200	0.225	50.0	45	2250	50	400
6. Sephadex G-200	90	0.094	170	8.5	1440	31	1300
7. TEAE-cellulose	90	0.02	460	1.8	830	18	3000

This table is based on the yield from 2 fermenters of culture. The weight of the mycelial pad was approx. 1 kg.

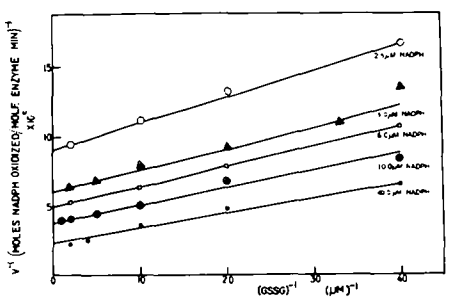
phoresis in either the ORNSTEIN AND DAVIS[26] or the SMITH AND HEDRICK[27] system. In both systems the protein was visible as a sharp yellow band even before staining.

Upon application to Sephadex G-200, it eluted as a single protein peak with constant specific activity in all fractions.

The enzyme moved as one peak upon sedimentation in the ultracentrifuge, and plots of log c *versus* x^2 from sedimentation equilibria data yielded a straight line.

Properties of glutathione reductase

The Michaelis constant. The K_m for each substrate was determined in two different ways. Figs. 1 and 2 represent the classic plot of a two-substrate system that adheres to the CLELAND[28] ping-pong bi-bi mechanism, in which the enzyme reacts first with one substrate to be converted to a modified form and then reacts with the second substrate, which converts it back to the original form. This agrees with the



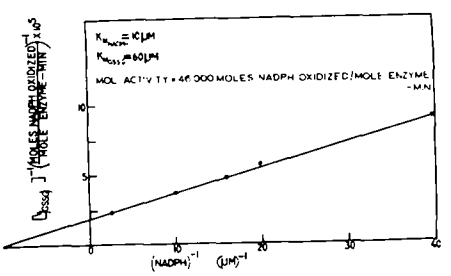


Fig. 1. Rate of glutathione reductase reaction as influenced by the concentration of substrates. Reactions were run at 25° in a Cary recording spectrophotometer using a 10-cm cell with a 5-cm light path.

Fig. 2. Effect of NADPH concentration on the V_{GSSG} values obtained for the experiment described in Fig. 1. Kinetic constants are determined according to DALZIEL[37].

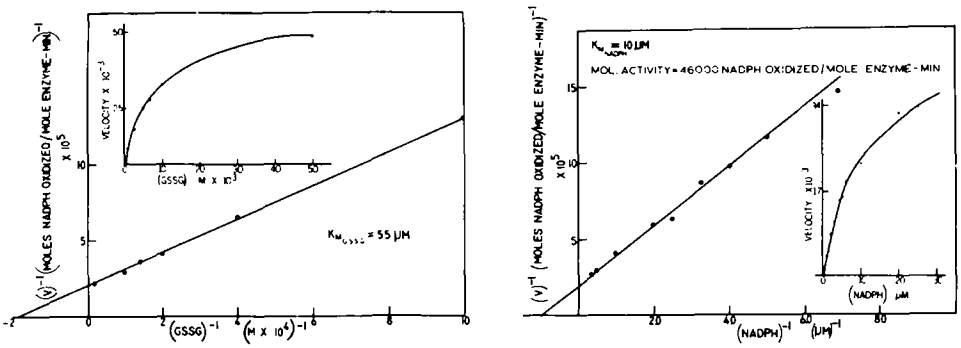


Fig. 3. Rate of the glutathione reductase reaction at saturating NADPH concentrations as influenced by the concentration of GSSG. Reactions were run at 25° in the Cary recording spectrophotometer using a standard 1-ml cuvette with a 1-cm light path. Assays were done in 1.0 ml of 0.1 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA, 0.2 μmole of NADPH and 0.04 μg of enzyme. Kinetic constants were determined according to LINEWEAVER AND BURK[38].

Fig. 4. Rate of glutathione reductase reaction at saturating GSSG concentrations as influenced by the concentration of NADPH. Reactions were run at 25°, in the Farrand recording spectrofluorometer (exciting wavelength 340 mμ, recording wavelength 460 mμ). Assays were performed in 3 ml of phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA, 15 μmoles of GSSG and 1.01 μg of enzyme.

results reported for glutathione reductase from other sources and fits the mechanism proposed by MASSEY[17].

Figs. 3 and 4 show that the K_m values for NADPH and GSSG at saturating concentrations of the other substrate are as predicted from the data in Figs. 1 and 2.

Since highly purified preparations of glutathione reductase from pea seedlings,

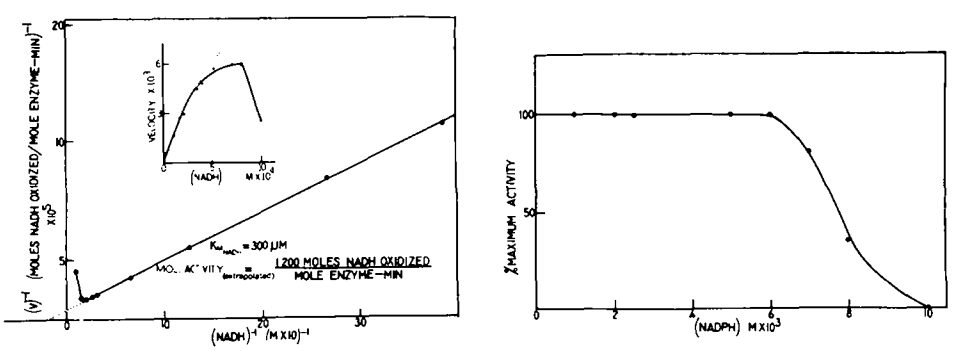


Fig. 5. Rates of glutathione reductase at saturating concentrations of GSSG as influenced by the concentration of NADH. Assays contained 3 μg enzyme, 5 μmoles GSSG and the indicated amounts of NADH in a volume of 1 ml 0.1 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA. The change in $A_{340\ m\mu}$ was recorded on a Cary recording spectrophotometer equipped with a 0–0.1 slide wire.

Fig. 6. Inhibition of glutathione reductase by high concentrations of NADPH. Reactions were run at 25° in the Cary recording spectrophotometer. Assays were done in 1 ml of 0.1 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA, 5 μmoles GSSG and enough enzyme (0.04–0.08 μg) to give a $\Delta A_{340\ m\mu}$/30 sec of 0.05 0.10 (uninhibited rate). Four assays were run for each NADPH concentration with duplicates at each of two enzyme concentrations. Where necessary, the assay solutions were read against blanks containing NADPH and buffer.

rat liver and human erythrocytes were reported to have slight activity with NADH, the K_m for NADH was also determined (Fig. 5). The results are comparable to those reported for glutathione reductase from other sources; glutathione reductase from both pea seedlings[4] and rat liver[20] has been reported to oxidize NADH at approx. 1% the rate it oxidizes NADPH. The v_{max} of glutathione reductase from human erythrocytes[21] with NADH as substrate has been reported as 115 μmoles/min per mg enzyme while the K_m for NADH was reported as 193 μM.

Effect of excess substrate. Concentrations of GSSG up to 0.1 M had no effect on glutathione reductase activity. However, the enzyme was inhibited by excess NADPH as shown in Fig. 6. This did not result from contamination of the NADPH by $NADP^+$ because at high (greater than 6 mM) concentrations of NADPH, the reaction rate increased as the NADPH was oxidized. As can be seen in Fig. 5, a similar behavior was observed with NADH. In this case, the concentration at which the inhibition became apparent was lower than that necessary to saturate the enzyme, and the observed reaction rate never reaches the theoretical (extrapolated) v_{max}.

Rat liver glutathione reductase[20] is reported to be 100% inhibited by 0.34 mM NADPH, but to be unaffected by high concentrations of GSSG. On the other hand, human erythrocyte glutathione reductase[21] is reported to be inhibited by high concentrations of either GSSG or NADPH. MASSEY AND WILLIAMS[17] offer the mecha-

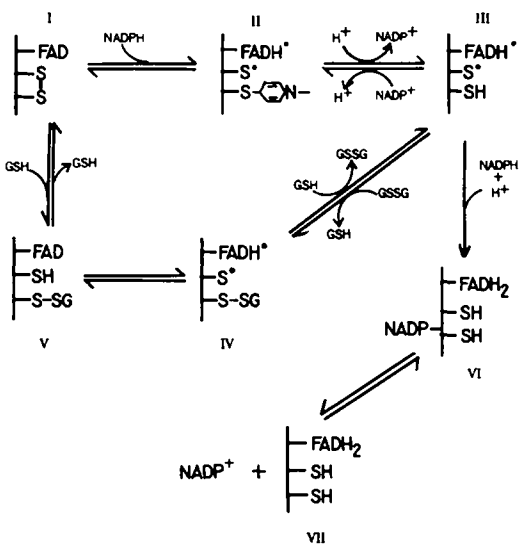


Fig. 7. Mechanism for yeast glutathione reductase.

nism shown in Fig. 7 for yeast glutathione reductase which is compatible with our observations. High concentrations of NADPH lead to the build-up of inactive forms VI and VII.

Effect of high concentrations of $NADP^+$. Since glutathione (reduced) (GSH) is easily autooxidized at pH 7.4 and all commercial samples we obtained were slightly contaminated with oxidized glutathione, no attempt was made to analyze the effect

of excess GSH on glutathione reductase. High concentrations of $NADP^+$ exhibited non-competitive inhibition when included in assays where either NADPH or GSSG were below saturating concentrations. The K_i for $NADP^+$ is 1.0–2.5 mM.

Effect of ionic strength. Glutathione reductase is influenced both by the ionic strength and the nature of the ions in the media in which it is assayed. In phosphate buffer, at pH 7.4, optimum activity is observed at 0.1 M. Additions of other salts to this solution decreases the activity of glutathione reductase. When assays were run in 0.01 M phosphate buffer, addition of other salts initially caused an increase in rate up to that observed in 0.1 M phosphate. With further increasing salt concentration, the reductase was inhibited. This was particularly striking with the halide ions whose effect on glutathione reductase activity was greatest with iodide and least with fluoride (Fig. 8). Bromide ions exhibited effects intermediate between those of chloride and iodide. Sodium sulfate exhibited effects similar to that of fluoride when added to 0.01 M phosphate.

Effect of metals and other inhibitors. As can be seen from Table II, glutathione reductase is not inhibited by short time incubation with cyanide, azide or iodoacetamide. It is inhibited by most thiol binding reagents but in a unique manner. Initial

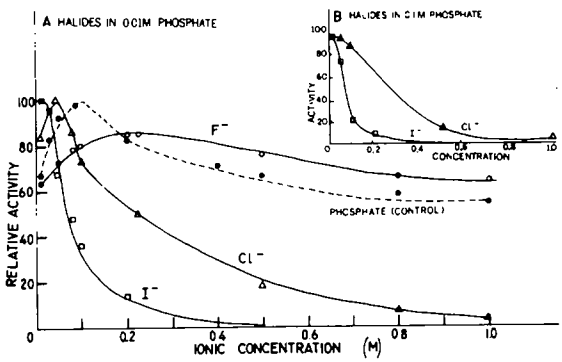


Fig. 8. Effect of ionic strength on the activity of glutathione reductase. Curve A: Standard assay conditions were used except that the buffer concentration was 0.01 M. For each assay mixture twice the desired salt concentration was added to 0.01 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA. The pH was readjusted to 7.4 with 0.01 M K_2HPO_4 or 0.01 M KH_2PO_4 and the volume adjusted appropriately with 0.01 M buffer. The effect of varying phosphate buffer alone is included for comparison. Curve B: Conditions were the same as in Curve A but the buffer used was 0.1 M phosphate.

velocities with thiol reagents present were low, but as the reaction proceeded and increasing amounts of GSH became available to compete for thiol binding inhibitors, the observed reaction velocity increased. Of the thiol binding inhibitors tested, PCMB was most effective and iodoacetamide the least. Preincubation of enzyme with inhibitor in the presence of either substrate had interesting results. Preincubation with NADPH increased the sensitivity of glutathione reductase to thiol binding reagents while GSSG had a protective effect. Such results have been noted before with the pea and yeast enzymes. When the enzyme was preincubated with inhibitor alone and the substrates were added serially to start the reaction, it was found that the

TABLE II

INHIBITION OF GLUTATHIONE REDUCTASE

Assays were run using standard concentrations of GSSG and NADPH in 0.1 M phosphate buffer (pH 7.4). Assays to determine the effect of heavy metals were run in 0.1 M *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid/2-(*N*-morpholino)ethane sulfonic acids (TES-MES) buffer (pH 7.4) using enzyme solutions which had previously been equilibrated with this buffer. Column I reports the inhibition rates when glutathione reductase and the indicated inhibitor were incubated for 3 min at 25° before the addition of NADPH and GSSG. Column II reports the results when the enzyme was incubated for 3 min at 25° with the indicated inhibitor and NADPH before the addition of GSSG. Column III reports the inhibition observed when the enzyme was incubated for 3 min at 25° with GSSG and the indicated inhibitor before the addition of NADPH.

Inhibitor	*Concn. of inhibitor (M)*	*% Inhibition*		
		I	*II*	*III*
PCMB	10^{-5}	100	100	100
	$5 \cdot 10^{-6}$	46	100	12
	$2.5 \cdot 10^{-6}$	37	100	00
	10^{-6}	17	100	00
	10^{-7}	00	95	00
Sodium azide	10^{-3}	00	00	00
Iodoacetate	10^{-2}	00	5	00
Iodoacetamide	10^{-2}	00	00	00
Sodium arsenite	10^{-2}	00	20	00
Sodium cyanide	10^{-2}	00	00	00
N-Ethylmaleimide	10^{-5}	00	16	00
	$5 \cdot 10^{-5}$	00	25	00
	10^{-3}	00	100	00
$CuCl_2$	10^{-5}	100	100	100
$AgNO_3$	10^{-6}	100	100	50
$BaCl_2$	10^{-6}	20	20	0
$ZnCl_2$	10^{-6}	0	100	0
$MgCl_2$	10^{-5}	0	0	0
$MnCl_2$	10^{-5}	0	0	0

sensitivity of the enzyme to thiol reagents was increased if NADPH was added before GSSG.

Preincubation of the enzyme with both heavy metals and EDTA resulted in rates equal to the uninhibited rates. EDTA added to the assay mixture after preincubation of heavy metal, substrate, and enzyme did not reverse the observed inhibition of the enzyme by the metal.

These results agree with the mechanism for yeast glutathione reductase advanced by MASSEY. The thiol essential for enzyme activity is not present unless the enzyme is first reduced by NADPH (form III, Fig. 7).

pH optimum. The pH optimum of the enzyme is broad, from pH 6.8 to 7.6. Results were the same with or without EDTA. Reported pH optima for glutathione reductase from other sources are recorded in Table V.

Equilibrium constant. The equilibrium constant of the reaction

$$NADPH + GSSG + H^+ \rightleftarrows NADP^+ + 2\,GSH$$

at various substrate concentrations is shown in Table III.

Using similar methods, MAPSON AND ISHERWOOD[4] reported a K_{eq} of $6 \cdot 10^{-13}$, while SCOTT, DUNCAN AND EKSTRAND[21] reported a K_{eq} of 10^{-9}.

TABLE III

EQUILIBRIUM CONSTANT FOR THE GLUTATHIONE REDUCTASE REACTION

$$K = \frac{(NADPH)\,(GSSG)\,(H^+)}{(NADP^+)\,(GSH)^2}$$

The indicated amounts of $NADP^+$ and GSH were incubated with glutathione reductase at 25° in 1 ml of 0.1 M phosphate buffer (pH 7.0). The increase in absorption at 340 mμ was followed on a Cary recording spectrophotometer equipped with a 0 0.1 slide wire.

Initial concn. of NADP⁺ (mM)	*Initial concn. of GSH (mM)*	*Equilibrium concn. of NADPH (μM)*	$K \times 10^{11}$
5	5	5.6	2.8
5	10	11.4	2.4
2.5	10	8.2	2.7

Spectra. The spectrum of the enzyme (Fig. 9) is similar to that reported for yeast[17,18], with peaks at 462, 365 and 275 mμ. The ratio of absorbance at 280 mμ to that at 460 mμ is 9.0–9.5. That reported for purified yeast glutathione reductase is 8.9–9.3. The $E^{1\%}_{1\,cm}$ at 230 mμ is 75. If the enzyme is frozen in dilute phosphate buffer (0.05 M or less), its spectrum upon thawing is slightly different and more nearly resembles that reported for pea seedling[4] and human erythrocyte[21] glutathione reductase. No observable turbidity resulted upon thawing. The two peaks above the

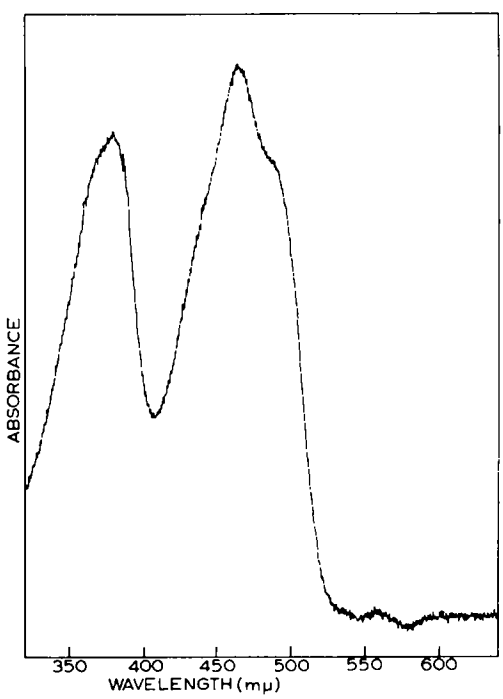


Fig. 9. Spectra of purified *P. chrysogenum* glutathione reductase (0.65 mg enzyme/ml of 0.1 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA) recorded on a Cary recording spectrophotometer equipped with a 0 0.1 slide wire.

ultraviolet region become broader and the 365-mμ peak becomes higher than the 462-mμ peak. The $E^{1\%}_{1\,cm}$ at 280 mμ also increases. No change in activity was observed to accompany the spectral changes.

When glutathione reductase is incubated anaerobically with an excess of NADPH, the typical semiquinone spectrum of glutathione reductase becomes apparent. The semiquinone intermediate has been noted in glutathione reductase from all sources studied in any detail.

There is some controversy over the $E^{1\%}_{1\,cm}$ at 280 mμ of yeast glutathione reductase. MASSEY AND WILLIAMS[17] report an $E^{1\%}_{1\,cm}$ at 280 mμ of 18.6 based on the biuret method using ribonuclease, rabbit muscle aldolase and chymotrypsin as standards. COLEMAN AND BLACK[18] obtained an $E^{1\%}_{1\,cm}$ at 280 mμ of 10.6 based on dry weight, while MASSEY AND WILLIAMS give a preliminary estimate of $E^{1\%}_{1\,cm}$ at 280 mμ $\geq$ 14.5 using the same method.

Using the biuret and LOWRY[29] procedures (with bovine serum albumin as the standard) and the 215–225-mμ absorption method, we obtained an $E^{1\%}_{1\,cm}$ at 280 mμ of 18.6.

Flavin content. Based on an $E^{1\%}_{1\,cm}$ at 280 mμ of 18.6 for glutathione reductase and a molar extinction coefficient of $1.13 \cdot 10^4$ for enzyme-bound flavin[17], it was determined that 1 mole of flavin is present per 55 000 g protein. The number of g protein per mole of flavin reported for glutathione reductase isolated from other sources is recorded in Table V. The data of MASSEY AND WILLIAMS seem to contradict that of COLEMAN AND BLACK. However, if COLEMAN AND BLACK's conclusion: that yeast glutathione reductase has 1 flavin per 118 000 g of protein is recalculated using an $E^{1\%}_{1\,cm}$ at 280 mμ of 18.6 (instead of 10.5), a value of 1 flavin per 67 000 g of protein is obtained.

The nature of the flavin moiety is not easy to ascertain. ASNIS[13] dissociated the flavin from *E. coli* glutathione reductase by precipitating the protein from a buffered solution with an equal volume of a saturated solution of ammonium sulfate, pH 0.6. BUZZARD AND KOPKO[30] dissociated the flavin from the rat liver enzyme by precipitating the protein from solution with an equal volume of saturated ammonium sulfate and titrating the solution to pH 4.0. The data from both groups show that, under the above conditions, flavin is incompletely dissociated from the protein and addition of FAD to the apoenzyme only partially restored complete activity. The addition of FMN or riboflavin had no or very little effect.

BLACK AND HUDSON[31] report difficulty dissociating FAD from the enzyme by the classical method of acid ammonium sulfate precipitation as used by BUZZARD AND KOPKO[30] and only partial (3%) restoration of activity when FAD is added to the apoenzyme prepared by the method of ASNIS[13].

To dissociate flavin from the *P. chrysogenum* enzyme a rather drastic method was used. As can be seen in Table IV, only FAD was effective in restoring activity, but the activity recovered is only 2% of the original.

Molecular weight. The molecular weight was determined by two separate methods; (a) the sedimentation equilibrium method of SCHACHMAN[32] using the formula

$$M_w = \frac{2RT\,(2.303)\,(\mathrm{d}\log c/\mathrm{d}x^2)}{(1 - \bar{V}\varrho)\omega^2}$$

TABLE IV

DISSOCIATION OF FLAVIN FROM *P. chrysogenum* GLUTATHIONE REDUCTASE

A sample of the enzyme (0.5 ml) was diluted with 0.5 ml water and 700 mg of ammonium sulfate were added to give 100% saturation. The crystals were dissolved by vigorous stirring and the pH adjusted to 3.0 with HCl. The precipitate was allowed to sit for 10 min and then centrifuged at high speed in an International Clinical Centrifuge in the 4° cold room. The supernatant solution was removed and the precipitate dissolved in 1 ml of 0.1 M PO_4 buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA, and again precipitated as above. The reprecipitation was then repeated. The protein at this stage still had some absorbance at 400 mμ and so it was precipitated with saturated $(NH_4)_2SO_4$ (pH 0.6). The precipitate was allowed to sit for 3 min, collected by centrifugation as above and then dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.4), containing $5 \cdot 10^{-4}$ M EDTA. Since much of the protein did not dissolve, the solution was again centrifuged before assay. The redissolved protein, designated as treated, undialyzed enzyme in Table IV, was assayed with and without added FAD and FMN and then split into 3 aliquots. 1 aliquot (dialyzed enzyme, Table IV) was dialyzed overnight against 0.1 M phosphate buffer. Another portion was dialyzed against 0.1 M phosphate buffer containing 50 μM FAD, and the third portion was dialyzed against 0.1 M phosphate buffer containing 50 μM FMN. Each sample was then assayed for activity and its protein concentration was determined from the absorption at 280 mμ using an $E^{1\%}_{1cm}$ at 280 mμ of 18.6. The protein concentration of the dialyzed samples was determined by measuring their absorption at 280 mμ and subtracting the absorbance of their respective dialysates.

Enzyme treatment	*Specific activity*	*% Original specific activity recovered*
Untreated enzyme	150	-
Treated undialyzed enzyme	0.05	0.03
Treated undialyzed enzyme *plus* FMN	0.05	0.03
Treated undialyzed enzyme *plus* FAD	1.5	1.0
Dialyzed enzyme	0.05	0.03
Dialyzed enzyme *plus* FMN	0.05	0.03
Dialyzed enzyme *plus* FAD	3.0	2.0

and (b) the method of ANDREWS[33] based on the elution volume of a protein from a calibrated Sephadex column. The calculated molecular weights were 110 000 based on the Sephadex data and 109 000 based on sedimentation equilibrium data.

$s_{20,w}$ values based on sedimentation velocity data, or on the sedimentation assay technique of COHEN AND HAHN[34] were determined from the equation

$$s_{20,w} = s_{obs}\left(\frac{\eta_t}{\eta_{20}}\right)_w\left(\frac{\eta}{\eta_0}\right)\left(\frac{1 - \bar{V}\varrho_{20(w}}{1 \quad \bar{V}(\varrho/\varrho_0)\varrho_{t,w)}}\right)$$

where

$$s_{obs} = \frac{2.303}{\omega^2 \cdot 60} \cdot \frac{d \log x}{dt_m}$$

The $s_{20,w}$ as determined by the standard sedimentation technique was $5.5 \cdot 10^{-13}$ sec, that determined by the sedimentation assay method was $5.6 \cdot 10^{-13}$ sec.

Diffusion coefficient. The diffusion coefficient was calculated from the sedimentation data using the formula

$$M = \frac{s_{20,w}RT}{D(1 - \bar{V}\varrho)}$$

and from the elution volume of glutathione reductase from the calibrated Sephadex

TABLE V

COMPARISON OF THE PROPERTIES OF GLUTATHIONE REDUCTASE FROM VARIOUS SOURCES

Source	*Mol. wt.*	*Method of determining mol. wt.*	*Method of protein determination*	*Specific activity*	*Flavin/ mole*	K_{mNADPH} *(μM)*	K_{mGSSG} *(μM)*	$D \times 10^7$	*pH optimum*	*Ref.*
Human erythrocytes	132 000	Based on an assumption of 1 mole flavin/mole protein and determined by g protein/mole flavin	$A_{280\ m\mu}$	182	1	16	96	—	6.8	21
Rat liver	44 000	Sedimentation velocity	$A_{280\ m\mu}$	839	1	3	50	9	7.0–8.5	20
Pea seedlings	60 000	1. Sedimentation velocity 2. g protein/mole flavin assuming 1 mole flavin/ mole protein	Lowry or absorption at 460 mμ	270	1	4.7	17		7.4	4
Yeast	113 000 123 000	High-speed sedimentation equilibrium		87	1	–	55	4.65		18
Yeast	56 500	Based on 1 flavin/mole enzyme		295	1	3.8	55	—	6.8–7.5	17
E. coli	—	– –	—	– –	—	37	1400	– –	6.9	13
P. chrysogenum	110 000	1. Calibrated Sephadex G-200		400	2	10	55	5.2	7.0–7.6	
	109 000	2. Sedimentation equilibrium								
	110 000	3. Based on 2 flavins/mole enzyme								

G-200 column, using the method of ACKERS[35] as given by ROGERS, HELLERMAN AND THOMPSON[36].

The diffusion coefficient calculated from sedimentation data was $5.15 \cdot 10^{-7}$ cm^2/sec, that calculated from Sephadex data was $5.2 \cdot 10^{-7}$ cm^2/sec. Using the data of BLACK AND COLEMAN[18] and the equation $M = s_{20,w} RT/D(1 - \bar{V}\varrho)$, we calculated that the diffusion coefficient of yeast glutathione reductase is $4.65 \cdot 10^{-7}$ cm^2/sec, while MIZE AND LANGDON[20] report a diffusion coefficient of $9 \cdot 10^{-7}$ cm^2/sec for glutathione reductase isolated from rat liver.

The striking similarity of the glutathione reductase isolated from various sources can be seen from the data summarized in Table V.

ACKNOWLEDGEMENTS

This work was supported by U.S. Public Health Service Research Grant GM-12292, U.S. Public Health Service Training Grant 5T1-6M-119 (T.S.W.), and National Science Foundation Reserarch Grant GB-5376.

Dr. GARY HATHAWAY and Dr. JOAN WILLEMOT assisted in obtaining the analytical ultracentrifuge data. Mr. DON DEVINCENZI kindly supplied the calibrated Sephadex column. Mr. ALAN SMITH performed the disc gel electrophoresis. Miss TRUDY WOOD was of great assistance during purification of the enzyme.

REFERENCES

1 E. E. CONN AND B. VENNESLAND, *Nature*, 167 (1951) 978.
2 L. W. MAPSON AND D. R. GODDARD, *Nature*, 167 (1951) 975.
3 E. E. CONN AND B. VENNESLAND, *J. Biol. Chem.*, 192 (1951) 17.
4 L. W. MAPSON AND F. A. ISHERWOOD, *Biochem. J.*, 86 (1963) 173.
5 T. W. RALL AND A. L. LEHNINGER, *J. Biol. Chem.*, 194 (1952) 119.
6 R. VAN HEYNINGEN AND N. W. PIRIE, *Biochem. J.*, 53 (1953) 436.
7 T. FUJIMOTO, *Am. J. Ophthalmol.*, 36 (1953) 737.
8 E. RACKER, *J. Biol. Chem.*, 217 (1955) 855.
9 R. G. LANGDON, *Biochim. Biophys. Acta*, 30 (1958) 432.
10 H. HORN AND E. H. BRUNS, *Biochem. Z.*, 331 (1959) 58.
11 M. FRANCOEUR AND O. F. DENSTEDT, *Can. J. Biochem. Physiol.*, 32 (1954) 663.
12 H. G. WILLIAMS-ASHMAN, *Cancer Res.*, 13 (1953) 721.
13 R. E. ASNIS, *J. Biol. Chem.*, 213 (1955) 77.
14 I. SUZUKI AND C. H. WERKMAN, *J. Biol. Chem.*, 240 (1965) 4470.
15 R. G. EAGON, *J. Bacteriol.*, 84 (1962) 819.
16 R. P. PAWNING AND H. IRZYKIEWICZ, *Austr. J. Biol. Sci.*, 13 (1960) 59.
17 V. MASSEY AND C. H. WILLIAMS, *J. Biol. Chem.*, 240 (1965) 4470.
18 R. F. COLEMAN AND S. BLACK, *J. Biol. Chem.*, 240 (1965) 1796.
19 D. J. D. NICHOLAS, A. NASON AND W. D. MCELROY, *J. Biol. Chem.*, 207 (1954) 341.
20 E. E. MIZE AND R. G. LANGDON, *J. Biol. Chem.*, 237 (1962) 1589.
21 E. M. SCOTT, I. W. DUNCAN AND V. J. EKSTRAND, *J. Biol. Chem.*, 238 (1963) 3928.
22 T. S. WOODIN AND I. H. SEGEL, *Biochim. Biophys. Acta*, 164 (1968) 78.
23 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 162 (1968) 78.
24 W. J. WADDELL, *J. Lab. Clin. Med.*, 48 (1956) 311.
25 P. V. BENKO, T. C. WOOD AND I. H. SEGEL, *Arch. Biochem. Biophys.*, 122 (1967) 783.
26 B. J. DAVIS AND L. ORNSTEIN, *Disc Electrophoresis*, Canalco, Bethesda, Md., 1962.
27 A. SMITH AND J. HEDRICK, *Arch. Biochem. Biophys.*, in the press.
28 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 104.
29 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
30 J. A. BUZZARD AND F. KOPKO, *J. Biol. Chem.*, 238 (1963) 464.
31 S. BLACK, *Ann. Rev. Biochem.*, 32 (1963) 399.

32 H. K. SCHACHMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 4, Academic Press, New York, p. 32.
33 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
34 R. COHEN AND C. HAHN, *Compt. Rend.*, 13 (1965) 2077.
35 G. K. ACKERS, *Biochemistry*, 3 (1964) 723.
36 K. S. ROGERS, L. HELLERMAN AND T. E. THOMPSON, *J. Biol. Chem.*, 240 (1965) 198.
37 K. DALZIEL, *Acta Chem. Scand.*, 4 (1957) 715.
38 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
39 M. DIXON, *Biochem. J.*, 55 (1953) 170.