On the Reaction Mechanism of Yeast Glutathione Reductase*

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Glutathione reductase has been purified from a number of sources, *Escherichia coli* (1), yeast (2, 3), peas (4), rat tissues (5, 6), and erythrocytes (7). In all of the cases, the enzyme appears to be a flavoprotein, containing flavin adenine dinucleotide as prosthetic group (8). In view of the fact that the over-all reaction catalyzed by the enzyme, GSSG + TPNH + H+ → 2 GSH + TPN+, is formally so similar to that catalyzed by lipoyl dehydrogenase, another flavoprotein enzyme, oxidized lipote (−S−S−) + DPNH + H+ → reduced lipote (−SH2) + DPN+, it is of obvious interest to determine whether the two enzymes have similar reaction mechanisms. In the case of pig heart lipoyl dehydrogenase, it has been found that a protein disulfide linkage (9–11) is concerned in catalysis, in close concert with the FAD prosthetic group. The anaerobic addition of reducing substrate (either DPNH or dihydrolipoyl derivatives) results in the formation of a stable two-electron reduction intermediate which has been shown to be extremely important in catalysis (12). This intermediate appears to be a complex between the enzyme flavin semiquinone and a sulfur radical formed from the active center disulfide (13). The intermediate has a very characteristic absorption spectrum, quite different from that found with half reduced forms of other flavoproteins. Our interest in the possible similarity of lipoyl dehydrogenase and glutathione reductase was considerably aroused by the published spectra of yeast glutathione reductase (3) which showed characteristics very similar to those found with lipoyl dehydrogenase. It was suggested therefore that the two enzymes may have very similar reaction mechanisms (14), i.e. that in glutathione reductase a protein disulfide active center might also be operative. This suggestion appeared to have been discounted by later results of Colman and Black (15) who showed by experiments with ¹⁴C-labeled N-ethylmaleimide that only 1 sulfhydryl group was liberated on treatment of the enzyme with TPNH. In this paper, we wish to report on some studies with glutathione reductase which indicate that this enzyme does in fact possess a basically identical reaction mechanism to that of lipoyl dehydrogenase.

**Materials and Methods**

**Enzyme Assay**—For routine assays, the method used was essentially that described by Raeker (2). In a total volume of 1.0 ml, semimicrowells contained the following: 60 μmoles of sodium phosphate buffer, pH 7.6, 0.1 μmole of TPNH, 3.25 μmoles of oxidized glutathione, 3 μmoles of EDTA, and 2 mg of crystalline bovine serum albumin. The cuvette was incubated at 25°C, and reaction was begun by the addition of a suitable amount of enzyme. The decrease in absorbance at 340 μm was followed at 25°C with a Gilford recording spectrophotometer. Activity is defined as micromoles of TPNH oxidized per min per mg of protein. Routinely, protein concentration was estimated by absorbance at 280 μm with the value of ε₂₈₀ of 1.86 for a solution containing 1 mg of protein per ml.

The latter value was determined with the chromatographically purified enzyme, with the biuret method calibrated with ribonuclease, rabbit muscle aldolase, and chymotrypsinogen as standards. These three proteins in the standard 3-ml test volume yielded an absorbance at 540 μm of 0.120 ± 0.002 (see "Discussion" for further consideration of this point). In kinetic experiments, the concentrations of reactants were varied as described, and the results were calculated as turnover numbers, i.e. moles of TPNH oxidized per min per mole of enzyme flavin.

**Flavin Analysis**—Flavin was liberated from the enzyme by heating in neutral solution for 3 min in a boiling water bath. After centrifugation, the flavin content was determined fluorimetrically with an Amino-Bowman spectrophotometer, exciting wave length at 470 μm, emission wave length at 530 μm. *Naja naja* venom (Ross Allen Reptile Institute) was used as a source of phosphodiesterase for the conversion of FAD to FMN.

**Sulfhydryl Analysis**—Sulfhydryl groups were determined by amperometric titration with phenyl mercuric acetate according to the method described by Allison and Cecil (16). A Sargent model 3 polarograph with a thermostated microelectrolysis vessel was used in conjunction with a dropping mercury electrode.

**Anaerobic Spectra**—The spectra reported were obtained under anaerobic conditions with specially constructed cells with either two or four side arms from which sequential additions were made. Spectra were recorded with a Cary model 14 recording spectrophotometer, generally with a 0 to 0.1 absorbance slide wire. Anaerobiosis was obtained by at least four cycles of evacuation with a water pump and flushing with oxygen-free helium, the last stage being evacuation.

**Reagents**—"Crystalline" yeast glutathione reductase was obtained from Boehringer and further purified as described under "Results." *Neurospora* DPNase was prepared by the method of Kaplan (17). TPNH was obtained from the Sigma Chemical Company, oxidized glutathione from Boehringer, reduced glutathione from British Drug Houses, and crystalline bovine serum albumin from Armour. The phenyl mercuric acetate used was the microanalytical standard of Hopkins and Williams Ltd.

* The DPNase was prepared by Mrs. Joan K. McAllister.
England. p-Chloromercuriphenylsulfonate was obtained from Sigma. Glass-distilled water was used in the preparation of all solutions. FAD and FMN used in fluorimetric studies were chromatographically purified as described previously (18).

RESULTS

Purification of Glutathione Reductase—Crystalline glutathione reductase from Boehringer, 100 mg, was dissolved in 10 ml of 0.1 M phosphate, pH 7.6, containing 3 x 10^-4 M EDTA. The resulting solution had appreciable flavin fluorescence, and from its spectrum was clearly contaminated with heme protein or proteins (Fig. 1). The solution was dialyzed for 3 hours against three changes of 1-liter volumes of glass-distilled water and applied to a column of calcium phosphate gel (2 x 20 cm) suspended on cellulose (see Reference 19 for details of this method). The column was developed with 0.05 M phosphate, pH 7.6, containing 3 x 10^-4 M EDTA. This caused separation into three colored bands. Running fastest was a pink band, followed by the bulk of the yellow color. There was no flavin fluorescence associated with this yellow band. Still adsorbed tightly at the top of the column was a narrow yellow band which was highly fluorescent and which was identified as lipoyl dehydrogenase. Elution with 0.05 M phosphate was continued until the pink band was completely eluted; by this time the faster moving yellow band had diffused over the top half of the column. This band was next eluted in a volume of 70 ml with 0.1 M phosphate, pH 7.6, containing 3 x 10^-4 M EDTA and identified as the glutathione reductase. The lipoyl dehydrogenase was still tightly adsorbed at the top of the column and was eluted with 0.1 M phosphate, pH 7.6, containing 3 x 10^-4 M EDTA and 4% (w/v) (NH_4)_2SO_4.

The glutathione reductase fraction was concentrated by precipitation with 80% saturation (NH_4)_2SO_4 (56 g/100 ml of eluate), and the bright yellow precipitate was dissolved in 0.1 M phosphate, pH 7.6, containing 3 x 10^-4 M EDTA. Table I

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Spectra of commercial crystalline glutathione reductase before and after chromatography on a column of calcium phosphate gel suspended on cellulose. The spectrum of the purified enzyme has been corrected for the volume change occurring during purification (see Table I and text for details).

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume</th>
<th>( \Sigma D_m (\text{mol} \times \text{Volume}) )</th>
<th>( \Sigma D_m (\text{mol} \times \text{Volume}) )</th>
<th>Total units</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boehringer enzyme applied to column</td>
<td>10</td>
<td>134</td>
<td>8.25</td>
<td>7800</td>
<td>78</td>
</tr>
<tr>
<td>Heme fraction eluted with 0.05 M phosphate, pH 7.6</td>
<td>148</td>
<td>40.8</td>
<td>16.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glutathione reductase fraction eluted with 0.1 M phosphate, pH 7.6</td>
<td>70</td>
<td>40</td>
<td>4.55</td>
<td>6340</td>
<td>295</td>
</tr>
<tr>
<td>Lipoyl dehydrogenase fraction, eluted with 0.1 M phosphate, pH 7.6, +4%, w/v, (NH_4)_2SO_4 (also contains some glutathione reductase)</td>
<td>36</td>
<td>23.7</td>
<td>8.9</td>
<td>1050</td>
<td>82</td>
</tr>
<tr>
<td>Glutathione reductase fraction precipitated with 80% saturated (NH_4)_2SO_4</td>
<td>5.6</td>
<td>38.6</td>
<td>9.3</td>
<td>5030</td>
<td>270</td>
</tr>
</tbody>
</table>
shows analytical details of the purification, and Fig. 1 shows the spectrum of the purified enzyme compared with the starting material. The specific activity of the purified enzyme is some 10 times greater than that originally reported by Racker (2).

Flavin Analysis—In subsequent work, the extinction coefficient of the enzyme-bound flavin at 460 nm has been taken as equal to that of free FAD at 448 nm (1.13 x 10^4 10^{-3} cm^{-1}). This is based on the following analysis. The absorbance of the concentrated purified enzyme at 460 nm was 0.74, which on the above assumption would correspond to a concentration of flavin of 6.55 x 10^{-5} M. This was diluted 60-fold in 0.1 M phosphate, pH 7.0, to give a concentration of 1.09 mM and heated in a sealed tube (protected from light) for 3 min in a boiling water bath. After centrifugation, the fluorescence was found to be equivalent to 1.10 mM chromatographically purified FAD. Furthermore, on addition of a few milligrams of Naja naja venom, there was an 11-fold increase in fluorescence consistent with the conversion of FAD to FMN by the venom phosphodiesterase. As with the glutathione reductases from other sources (1, 4, 5) and in agreement with the results of Black (8), the prosthetic group of the yeast enzyme is thus identified as FAD. On the basis of the flavin content and the protein concentration of the purified enzyme (3.7 mg per ml), it is calculated that the minimum molecular weight of the yeast enzyme is 56,500 g per molecule of FAD. As Colman and Black (15) have estimated the molecular weight to be 118,000 by sedimentation equilibrium measurements, it would thus appear that there must be 2 molecules of FAD per molecule of protein. This conclusion is at variance from that of Colman and Black who found 1 mole of FAD per mole of protein. However, it is interesting to note that with the enzyme from pea seedlings, Mapson and Isherwood (4) determined a molecular weight of 60,000 based on the flavin content.

![Fig. 2. Kinetic analysis of the reduction of GSSG by TPNH catalyzed by glutathione reductase at 25°. Reaction conditions: 0.067 M phosphate, pH 7.6, in the presence of 10 mM EDTA and 0.007% crystalline bovine serum albumin and the indicated concentrations of TPNH and GSSG. The results with 8.5 x 10^{-4} M TPNH were obtained with a Gilford recording spectrophotometer with 1-cm light path cells; those with 1.43 mM TPNH with a Cary model 14 recording spectrophotometer with 10-cm light path cells.](image)

![Fig. 3. Variation of the extrapolated turnover number (at infinite concentration of GSSG) from experiments similar to those described in Fig. 2 at various concentrations of TPNH.](image)

Catalytic Activity—Yeast glutathione reductase has been found to exhibit the same type of kinetic behavior as shown first with lipoic dehydrogenase (12) and now found with a wide variety of flavoproteins (see Reference 14 for a review). Figs. 2 and 3 show the effect on activity of varying the concentrations of both oxidized glutathione and TPNH at pH 7.6. From these results, the maximum turnover number is calculated to be 15,000 moles of TPNH oxidized per min per mole of enzyme-bound FAD with K_{GSSG} = 5.5 x 10^{-4} M and K_{TPNH} = 3.8 mM. A similar kinetic behavior had previously been demonstrated with the enzyme from pea seedlings (4) with an almost identical maximum velocity but slightly different K_m values. This kinetic behavior (parallel Lineweaver-Burk plots) is typical of an enzyme reaction where the enzyme reacts with one substrate to be converted to a modified form before reacting with the second substrate which reconverts it to the original form. Fig. 4 shows that the enzyme-catalyzed reaction has a rather broad pH optimum centered around pH 7.1.

Effect of Reduced Glutathione on Spectrum—The spectra published by Black and Hudson (3) indicate that GSH reduces the enzyme in a very similar way to that found for reduction of lipoic dehydrogenase by dihydrolipoic derivatives (13). This is borne out by our work also. Fig. 5 shows the results of an anaerobic titration of yeast glutathione reductase with GSH. In keeping with the high oxidation-reduction potential of the glutathione couple, considerable molar excess of GSH is required to produce the full amount of the intermediate. The set of four isosbestic points seen in Fig. 5 indicate that, up to a molar ratio of 276 moles of GSH per mole of enzyme flavin, no significant quantities of species other than the oxidized enzyme and the intermediate reduced form exist. With 526 moles of GSH, some indication is seen of reduction beyond the stage of the long wave length absorbing species as evidenced by the fact that the isosbestic points are no longer perfect. The spectral characteristics of the intermediate mimic in remarkable detail those found on reduction of lipoic dehydrogenase (for comparison see Fig. 8 of Reference 13).

Effect of TPNH on Spectrum—In the case of lipoic dehy-
drogenase, the spectral effects of adding DPNH are complicated by at least two absorbing species in addition to the major component (which is the same as that produced by adding dihydrolipoyl derivatives). With the latter enzyme, reduction by DPNH leads to the formation of DPN which can form a charge transfer complex with fully reduced enzyme absorbing maximally at 720 m\(\mu\) (20) or with the half-reduced enzyme absorbing maximally around 580 m\(\mu\) (21). Therefore, small amounts of these species give a broader and flatter absorption in the spectral region above 500 m\(\mu\) than do dihydrolipoyl derivatives (20). The effects of these DPN complexes may be removed by titration with 1.0 mole of DPNH in the presence of Neurospora DPNase, which hydrolyses the DPN formed (13). Under these conditions, a quantitatively identical effect is obtained as with dihydrolipoyl compounds. The addition of more than 1.0 mole of DPNH in the presence of DPNase results in further reduction, to produce the fully reduced enzyme by 2.0 moles of DPNH per mole of enzyme flavin (13). This provided one of the most convincing pieces of evidence for the existence of the two prosthetic groups of lipoil dehydrogenase, since four reducing equivalents were required to reduce fully the flavin and disulfide prosthetic groups. That a similar phenomenon existed with glutathione reductase was suggested by the spectra published by Black and Hudson; reduction by TPNH resulted in a much broader absorption band above 500 m\(\mu\) than did GSH (3). It was therefore of considerable interest to determine the effect of anaerobic titration of glutathione reductase with TPNH in the presence of Neurospora DPNase. (The latter enzyme has been found to hydrolyze TPN as well as DPN (17).) When glutathione reductase was reacted with TPNH in the absence of DPNase, results similar to those found by Black and Hudson (2) were obtained (see Fig. 11 for comparison). When the reaction was carried out with approximately 1 mole of TPNH in the presence of DPNase, the resulting spectrum was within experimental error identical to that produced by GSH (Fig. 6). As with lipoil dehydrogenase, it was also found that in the presence of DPNase, excess TPNH gave full reduction with complete elimination of the long wave length absorption; in the absence of DPNase even an 11-fold excess of TPNH maintained undiminished the long wave length absorption. Fig. 7 shows the spectra obtained on reaction with TPNH in the presence of DPNase. Up to a nominal 1.25 moles of TPNH per mole of flavin, only the intermediate spectrum was obtained as evidenced by the same four isosbestic points as seen on titration with GSH (cf. Fig. 5). With larger amounts of TPNH further reduction clearly took place. Fig. 8 shows the extinction changes at 530 m\(\mu\) produced by various amounts of TPNH, and compares the results obtained in the presence and absence of DPNase. Qualitatively the results are very similar to those obtained with lipoil dehydrogenase and DPNH (see Fig. 4 and Reference 10). Quantitatively, however, the results...
Fig. 6. Comparison of the spectral changes induced by anaerobic addition to yeast glutathione reductase (—) of 598 moles of GSH per mole of enzyme flavin (— — —), and 1.25 moles of TPNH in the presence of 1100 Kaplan units of Neurospora DPNase (○-○). Results obtained in 0.067 M phosphate, pH 7.6, 25°.

Fig. 7. Titration of glutathione reductase under anaerobic conditions with TPNH in the presence of 1100 Kaplan units of Neurospora DPNase. The results were obtained in 0.067 M phosphate, pH 7.6, 25°. The spectra shown were recorded when no further changes had occurred as a function of time. No correction has been made for unreacted TPNH.

are not so clear-cut. With lipoyl dehydrogenase in the presence of DPNase, full (4-electron) reduction was obtained with 2.0 moles of DPNH. In the present experiments with glutathione reductase, more TPNH was required for full reduction. The actual amount has not yet been determined, but it is greater than 2 moles and less than 11 moles of TPNH per mole of enzyme flavin. These results may indicate a somewhat lower oxidation-reduction potential of the glutathione reductase than of lipoyl dehydrogenase. However, we are not yet convinced that this is the explanation; the samples of TPNH we have used have a not unappreciable instability at pH 7.6 and 25°, which appears to explain at least in part the differences between the results with the two enzymes. This problem of instability is aggravated by the slowness with which the full reduction in the presence of DPNase occurs. This is illustrated in Fig. 9 where analogous experiments were run side by side with lipoyl dehydrogenase and glutathione reductase. It can be seen that the rate of conversion of the intermediate to fully reduced enzyme in the presence of DPNase is slower by at least an order of magnitude for glutathione reductase than is the case with lipoyl dehydrogenase.
The possible significance of this finding will be considered further under "Discussion."

Effect of Arsenite. In the presence of arsenite, excess DPNH reduces lipoic dehydrogenase to the fully reduced state (both FAD and active center disulfide reduced fully); the DPN formed in this reaction combines with the enzyme to give a charge transfer complex with an absorption maximum at 720 μm (20). That this complex is between the FADH2 form of the enzyme and DPN was shown by the fact that Neurospora DPNase abolished the 720 μm band and gave the spectrum of fully reduced enzyme, such as is obtained with excess dithionite (20). Entirely analogous behavior was found with glutathione reductase that was allowed to react with excess TPNH in the presence of arsenite. Fig. 10 shows that under these conditions the same 720 μm absorption band is produced and that this is abolished in the presence of DPNase. When TPN was added after full reduction in the presence of DPNase, the 720 μm band was reformed.

Effect of Mercuroial on Spectrum.—With lipoic dehydrogenase, one of the first indications that the stable long wave length intermediate involved some interaction of flavin and a protein...
**Table II**

**Amperometric titration of sulfhydryl groups of glutathione reductase**

In each titration, carried out in 0.04 M borate, pH 9.0, 25°C, 2 ml of a solution of glutathione reductase, 1.3 × 10⁻⁵ M with respect to FAD, were used. Phenyl mercuric acetate (6 μM) was added from a microsyringe until an accurate end point was obtained and additions continued until at least another 10-μl volume had been added. In Experiments 1 to 4, 25 μl of 0.1 M TPNH was then added; in Experiment 5, 0.1 ml of 1 M Na₂SO₃ was added instead of the TPNH. In Experiment 4, where the enzyme had been denatured, the addition of TPNH was without effect. In Experiments 1 to 3, the addition of TPNH produced an immediate fall in the electrode current to the base value. In Experiment 5, the addition of sulfite produced the same effect. The extra sulfhydryl groups released by the addition of TPNH or sulfite were then titrated by addition of more phenyl mercuric acetate. In Experiments 1 and 2, the values for rapidly reacting —SH groups were calculated from the volume of mercurial at which the electrode current began to rise; the final values were obtained by extrapolation of the slope of current increase with titrant volume to the basal current. In all of the experiments, the mercurial was added in 2-μl quantities, and 4 to 5 min was allowed for reaction before the current was read and the next addition made.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>—SH groups titrated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Borate, pH 9</td>
<td>1.0</td>
</tr>
<tr>
<td>+ TPNH</td>
<td>3.4 fast, 4.05 final</td>
</tr>
<tr>
<td>2. Urea, 4.25 M</td>
<td>1.1</td>
</tr>
<tr>
<td>+ TPNH</td>
<td>4.5 fast, 6.0 final</td>
</tr>
<tr>
<td>3. Urea, 6.35 M</td>
<td>1.1</td>
</tr>
<tr>
<td>+ TPNH</td>
<td>5.4</td>
</tr>
<tr>
<td>4. Urea, 9 M</td>
<td>3.5</td>
</tr>
<tr>
<td>+ TPNH</td>
<td>3.5</td>
</tr>
<tr>
<td>5. Urea, 8.5 M</td>
<td>3.45</td>
</tr>
<tr>
<td>+ sulfite</td>
<td>5.05</td>
</tr>
</tbody>
</table>

*The values quoted refer to moles of sulfhydryl per mole of enzyme-bound FAD.

The sulfur atom came from the finding that organic mercurials caused the disappearance of this species and production of the spectrum of fully reduced enzyme. Again almost identical results were obtained with glutathione reductase (Fig. 11). Whereas in the absence of mercurial, the spectral shift induced by TPNH was almost indefinitely stable under anaerobic conditions, the addition of p-chloromercuriphenylsulfonate led to the fairly rapid disappearance of the long wave length absorption; the resulting spectrum was clearly that of enzyme in which the FAD was fully reduced.

**Protein Sulfhydryl Estimations**—In view of the striking similarities between lipoyl dehydrogenase and glutathione reductase already noted, it was of obvious interest to determine directly the number of —SH groups present in the enzyme and the effect of TPNH on the measured —SH content. Table II summarizes the results of a number of experiments involving amperometric titration of the sulfhydryl groups of the enzyme. The results are complicated somewhat by the finding of rapidly reacting and slowly reacting sulfhydryl groups. In Experiment 1, where no urea was employed, only 1 —SH group was detected in the native enzyme. When TPNH was added, the —SH titer rose immediately to 3.4, and finally reached the value of 4.05. In 4.25 M urea, only 1 sulfhydryl group was titrated; when TPNH was added, the titer rose immediately to 4.5, and finally reached the value of 6.0. In 6.35 M urea, again only 1 —SH group was titrated; addition of TPNH caused a rapid rise to a titer of 5.4. In 9.0 M urea, 3.5 —SH groups were titrated, presumably due to a concentration of urea now being used which leads to gross changes in the protein structure. Consistent with this, the subsequent addition of TPNH was now without effect, indicating that the enzyme had been denatured by 9 M urea but not by 6.35 M urea. Finally in Experiment 5, sulfite was substituted for TPNH in order to estimate disulfide by a nonsubstrate method. It is well known that sulfite reacts with disulfides in the following manner.


\[ R-S-S-R' + SO_3^- \rightleftharpoons RS^- + R'SSO_3^- \]

In the presence of a mercaptide-forming agent, such as phenyl mercuric acetate, this equilibrium is shifted all the way to the right. Thus, for every disulfide bond broken by sulfite, the sulfhydryl titer should increase by 1. A schematic interpretation of the results of Table II is given under “Discussion.”

**DISCUSSION**

The experiments described leave little doubt that there is a remarkable similarity in properties between glutathione reductase and lipoyl dehydrogenase, and that all of the properties of the latter enzyme, indicating the reversible oxido-reduction of a protein disulfide in catalysis, are shown also by glutathione reductase. It should be emphasized of course that the two enzymes are different in several respects. Lipoyl dehydrogenase reacts much more rapidly with DPNH than with TPNH; the converse is true for glutathione reductase. The disulfide substrate specificity is absolute; lipoyl dehydrogenase is not active with oxidized glutathione, and glutathione reductase shows no reactivity with oxidized lipoic acid or derivatives. Lipoyl dehydrogenase is very fluorescent whereas yeast glutathione reductase is nonfluorescent. The two enzymes also differ in their sensitivity to inhibition by mercurials. As well as the active center disulfide, lipoyl dehydrogenase has at least 1 sulphydryl group which is very sensitive to mercaptoethanol formation. Lipoyl dehydrogenase is in fact irreversibly inhibited when the two rapidly reacting sulphydryl groups are reacted with p-chloromercuribenzoate (12, 22). This sensitivity is not displayed at all by glutathione reductase. From Table II and the description of these experiments in the text, it can be seen that even in 6.3 M urea reaction of the one freely reacting sulphydryl group does not impair reduction by TPNH. Furthermore, when the active center disulfide was reduced by TPNH, and the sulphydryl groups were allowed to react with mercaptoethanol, considerable reactivation could be obtained by treatment with excess GSH followed by dialysis. When the enzyme used in the experiment of Fig. 11 was opened to air, the spectrum obtained immediately was very similar to that of free FAD with a maximum at 448 m\(\mu\). On addition of solid GSH and dialysis against 0.1 M phosphate, pH 7.6, the spectrum of original enzyme was regained with a maximum at 462 m\(\mu\). The recovery of native enzyme in this experiment was at least 80%.

With these differences however, the divergencies between the two enzymes appear to end and the similarities become much more impressive than the differences. The two enzymes appear to have similar molecular weights, 100,000 for lipoyl dehydrogenase (23) and a tentative value of 118,000 for yeast glutathione reductase (15). Both enzymes have FAD as prosthetic group, with 2 molecules of FAD per molecule of protein. In the case of lipoyl dehydrogenase, the active center disulfide has been shown to be a cystine bridge linking two identical peptide chains (23); no information on this point is as yet available with the glutathione reductase, although the stability of the enzyme to high concentrations of urea suggests that the disulfide probably conveys a large degree of stabilization to the tertiary structure of the protein. The glutathione reductase of pea seedlings has also been found to be stable in the presence of high concentrations of urea (4). The results of the amperometric titration experiments may be explained simply in terms of a scheme such as that shown in Fig. 12. The native form of the enzyme is envisaged as having one freely exposed sulphydryl group, the remaining three groups being unreactive. On reaction with TPNH in the absence of urea, four —SH groups titrate: the freely reactive group, two presumably from the nascent active center disulfide, and the fourth from a partial unfolding of the molecule. The renaturation results described in the previous paragraph clearly indicate that no very extensive denaturation could have occurred under these conditions. In 4.5 M urea, however, with the active center disulfide reduced, it is very reasonable to expect a complete unfolding of the molecule with the result that all six of the sulphydryl groups can be titrated in the presence of TPNH. If the enzyme is placed in 8.5 M urea solution, it would appear that denaturation results so that four sulphydryl groups should now react. The fact that experimentally, 3.5 —SH groups were allowed to react, may reflect a partial hindrance still to complete reaction of the remaining sulphydryl groups. As expected of a denatured enzyme, TPNH under those conditions does not lead to an increased sulphydryl titer, whereas sulfite, by rupturing the active center disulfide, does do so and yields the expected reaction of five sulphydryl groups.

The conclusion from the amperometric titration data that TPNH reduces an active center disulfide is fully consistent with the various spectral experiments described. The anaerobic titration data of Fig. 8 indicate that, as in lipoyl dehydrogenase, the stable substrate-produced intermediate is a 2-electron reduction form of the enzyme. The effect of mercurial on this intermediate (Fig. 11) indicates, as with lipoyl dehydrogenase, that this species involves interaction of both the flavin prosthetic group and a component of the active center disulfide. Further evidence for the production of a fully reduced (4-electron reduction) state of the enzyme comes both from the effect of arsenite (Fig. 10) and the effect of DPNase (Figs. 7 to 9). One interesting
The difference between lipoyl dehydrogenase and glutathione reductase is the speed with which DPNase exerts its effect on the conversion of the 2-electron reduced form to the 4-electron reduced form (Fig. 9). The amount of DPNase used was sufficient to have hydrolyzed all of the free DPN or TPN within minutes (this was checked with free DPN and TPN). Thus, the data indicate that DPN and TPN must be bound to the two enzymes rather strongly, and that the TPN complex of glutathione reductase is more stable than the DPN complex of lipoyl dehydrogenase.

In view of the close similarities between the two enzymes, it is therefore proposed that glutathione reductase has a very similar reaction mechanism to that of lipoyl dehydrogenase. Fig. 13 is a schematic representation of the reaction mechanism we wish to propose as a working model for future experiments. Catalysis is envisaged as proceeding through the sequence of states, I → II → III → IV → V → I. State II would represent the proposed TPN complex of the 2-electron reduced enzyme; its conversion to the fully reduced enzyme by excess TPNH in the presence of DPNase being envisaged as proceeding via the sequence, II → III → VI → VII. In catalysis, (in rapid equilibrium with II) is envisaged as reacting with a molecule of oxidized glutathione to undergo disulfide interchange reaction and produce IV, which by electron rearrangement through V could undergo another disulfide interchange reaction to split off the second molecule of GSH and reform the original oxidized enzyme. This scheme would explain the effect of arsenite on the spectrum, as arsenite, by combining with VI, would displace the reaction in the presence of excess TPNH to the fully reduced form. As TPN is still bound in State VI, a charge transfer complex between the two enzyme mechanisms to almost the same extent as the pyridine nucleotide and flavin coenzymes.

In conclusion, we wish to reiterate the possibility that protein disulfide groups may serve as oxidation-reduction active groups in many enzymes, particularly in the case of flavoproteins. In the case of lipoyl dehydrogenase and glutathione reductase, it would appear that the disulfide is merely a cystine bridge, presumably made more reactive by the array of amino acid residues peculiar to the individual enzymes. In the case of at least two metalloflavoproteins, dihydroorotic dehydrogenase (24) and xanthine oxidase (25), recent evidence suggests that a similar reactive disulfide exists, in which one of the sulfur atoms is bonded to inorganic iron, conveying to the linkage a peculiar lability to acid, resulting in the liberation of H2S when the enzymes are acidified. This property is shared by every known metalloflavoprotein and also by ferredoxin (26).

The spectral characteristics of the enzyme have been examined with reducing substrates under a variety of conditions and found to be remarkably similar to those of lipoyl dehydrogenase under analogous conditions. From the effects of reaction of the enzyme with TPNH in the presence of Neurospora DPNase, arsenite, or mercurials, it is concluded that glutathione reductase has a reactive disulfide as part of the active center in addition to the flavin. This conclusion is reinforced by the

**SUMMARY**

1. “Crystalline” yeast glutathione reductase from a commercial source was purified a further 4-fold by chromatography on a calcium phosphate gel-cellulose column. The purified enzyme contained flavin adenine dinucleotide as prosthetic group, with a minimum molecular weight of 56,500 based on the flavin content.

2. The spectral characteristics of the enzyme have been examined with reducing substrates under a variety of conditions and found to be remarkably similar to those of lipoyl dehydrogenase under analogous conditions. From the effects of reaction of the enzyme with TPNH in the presence of Neurospora DPNase, arsenite, or mercurials, it is concluded that glutathione reductase has a reactive disulfide as part of the active center in addition to the flavin. This conclusion is reinforced by the
results of direct estimation of sulphhydryl groups by amperometric titration.

3. On the basis of the analogy with lipoyl dehydrogenase, a reaction mechanism for glutathione reductase is proposed.

Addendum—Since this paper was submitted for publication, a more detailed account of the results of Colman and Black has appeared (29). In several important respects, the results reported in that paper appear to differ from those reported here and it is felt both by Dr. Black and by us that some further discussion of these differences may be helpful in avoiding needless confusion. From the data given by Colman and Black (29), it may be calculated that the ratio of absorbances at 260 and 462 m of their enzyme is essentially the same as that reported in this paper (8.9 to 9.3). Although this agreement could be fortuitous (e.g. if their enzyme were contaminated with lipoyl dehydrogenase), it would therefore appear likely that we are dealing with enzyme at the same stage of purity and that many of the different conclusions reached can be explained in terms of analytical differences.

Absorbance at 280 m—Colman and Black obtained an ENm value of 10.5 by dry weight analysis, whereas we obtained a value of 18.6 by the biuret method calibrated against three proteins (ribonuclease A, rabbit muscle aldolase, and chymotrypsinogen). These three proteins gave a constant biuret coefficient (ENm* mg of 0.120 ± 0.002 in the standard 3 ml assay). However, in the same test, two other proteins, crystalline bovine serum albumin and crystalline yeast alcohol dehydrogenase, gave ENm* mg values of 0.085 and 0.095, respectively. Thus, it is clear that the attempted standardization of an “unknown” protein such as glutathione reductase by such a method is subject to considerable possible error. In principle, however, the standardization method used by Colman and Black (i.e. dry weight) is also subject to similar objections since this method, besides being subject to error through water retention or regain, also measures nonprotein material such as carbohydrate, lipid, and bound ions. One preliminary estimate carried out by us with the dry weight method yielded an ENm value of 14.5. Clearly, more experiments are required to reach a definitive answer to this point. However, one concrete point can be made concerning the apparent differences in specific activity between the two preparations. The value of 89 amoles min−1 mg−1 of Colman and Black is based on their ENm value of 10.5 and so should be multiplied by 18.6 to compare with our value. Thus, their enzyme would have a specific activity of 158 compared to our value of 270 to 295. The difference still existing could be due to a combination of factors, such as differences of temperature at which activity was measured and the presence of EDTA in our assay.

Unit Molecular Weight—By sedimentation equilibrium experiments, Colman and Black (29) have determined the molecular weight to be 118,000. On the basis of the flavin content and with the ENm value of 10.5, they calculated the unit molecular weight per g molecule of flavin to be 100,700, compared to our value of 56,500. Again it should be emphasized that this is an apparent difference only; if our ENm value of 18.6 is applied to their analyses, the unit molecular weight becomes 57,000. In an attempt to obtain a more definitive answer to this problem, and at the same time obtain an analytical value for total cysteine + cystine (see “Content of Cystine plus Cysteine”), a sample of enzyme was oxidized at -7°C for 10 hours with performic acid by the method of Hirs (30). Excess performic acid was destroyed by addition of water and freeze drying (twice); the freeze-dried material was hydrolyzed with 6 N HCl at 110°C for 18 hours in a sealed tube, and amino acid analysis was carried out by the method of Moore et al. (31, 32) with a Spinco automatic amino acid analyzer. The results, without any correction factors applied, are shown in Table III. From these results, which lack an analytical value for tryptophan, the molecular weight per g molecule of FAD is calculated to be 48,800. If correction factors for destruction of serine (1.09) and threonine (1.045) and incomplete liberation of valine (1.08) and isoleucine (1.16) are applied (Mahowald et al. 33, 34), the unit molecular weight estimate becomes 49,900. We thus believe that the enzyme of molecular weight 118,000 (29) contains two molecules of FAD.

Content of Cystine plus Cysteine—While the differences considered above are ones which revolve largely around two different estimates of the protein content per flavin, a more serious discrepancy is apparent in estimates of the sulphhydryl content of the enzyme. In our work, summarized in Table II, this was estimated by amperometric titration. It was found that, per molecule of FAD, the enzyme possessed one freely reactive sulphhydryl group, three —SH groups that were allowed to react only in the presence of high concentrations of urea, and one disulfide bond that could be reduced by TPNH or ruptured by sulfite. In contrast to these results, Colman and Black (29) with N-ethylmaleimide as titrant, estimated that per molecule of FAD, the enzyme contained one freely reactive —SH group, and that only two more —SH groups could be detected, one by reaction with TPNH, the other by exposure to 6 M urea. In

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar equivalents per mole of FAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteic acid</td>
<td>4.85</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>50.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.97</td>
</tr>
<tr>
<td>Serine</td>
<td>1.91</td>
</tr>
<tr>
<td>Proline</td>
<td>15.7</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>57.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>49.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>37.9</td>
</tr>
<tr>
<td>Valine</td>
<td>53.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>5.92</td>
</tr>
<tr>
<td>Methionine sulfone</td>
<td>5.87</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>32.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>40.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>9.34</td>
</tr>
<tr>
<td>Phenylnalanine</td>
<td>19.6</td>
</tr>
<tr>
<td>Ammonia</td>
<td>48.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>23.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>8.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.53</td>
</tr>
<tr>
<td>Molecular weight per mole of FAD</td>
<td>48,000</td>
</tr>
<tr>
<td>Plus FAD</td>
<td>785</td>
</tr>
<tr>
<td>Total</td>
<td>48,785</td>
</tr>
</tbody>
</table>
order to clarify this discrepancy, we subjected glutathione reductase to performic acid oxidation (to convert both cysteine and cystine to cysteic acid) and analyzed it for cysteic acid after acid hydrolysis, as described previously. Without any correction factors, 4.8 and 4.9 moles of cysteic acid were found per mole of FAD in two analyses. Estimates of recovery of cysteine and cystine as cysteic acid vary in the literature from 82 to 90% (33-36). Thus, the cysteine plus cystine content of glutathione reductase by the performic acid oxidation method is estimated to be within 5.4 to 5.9 units of cysteine eq per molecule of enzyme-bound FAD. Thus, the analyses are in good agreement with the results obtained by amperometric titration. It should be emphasized that the results shown in Table II in the presence of sulfite reveal the presence of one disulfide bridge per molecule of enzyme-bound FAD, and that in the presence of TPNH this disulfide can be titrated with mercurial as a dithiol. Taken in conjunction with the other results presented, we believe this to be convincing evidence for the presence of an active center disulfide in addition to the FAD prosthetic group.

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