GLUTATHIONE REDUCTASE FROM BAKERS' YEAST AND BEEF LIVER*

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The reduction of glutathione by a heat-labile system in liver was discovered by Hopkins and Elliott (1). Later Mann (2) obtained a soluble enzyme preparation from liver which required, for the reduction process, a cofactor and glucose as hydrogen donor. Meldrum and Tarr (3) found that oxidized glutathione was reduced by rat blood and by yeast and demonstrated the function of TPN¹ as a cofactor in this system. Hexose monophosphate was used as a hydrogen donor for the reduction of TPN. More recently Conn and Vennesland (4), Mapson and Goddard (5), and Rall and Lehninger (6) demonstrated the presence of glutathione reductase in wheat germ, pea seeds, and liver and showed that, in all instances, TPN was an obligatory cofactor and that DPN was inactive.

In the present communication the preparation of glutathione reductase from yeast and the partial purification of this enzyme from beef liver are reported. Under suitable conditions both enzyme preparations were found to be active with DPN as well as TPN as the coenzyme.

The reaction with reduced DPN is considerably slower than that with reduced TPN; it is stimulated by phosphate and inhibited by some other ions.

Results

Effect of Phosphate and Other Salts on Reduction of Oxidized Glutathione—When GSSG, TPN, or DPN and a suitable hydrogen donor system were added to a well dialyzed extract of dried bakers' yeast or to a partially purified preparation of yeast glutathione reductase, reduced glutathione was formed. Whereas in the presence of TPN as hydrogen transfer agent

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¹ The following abbreviations are used in this paper: TPN and TPNH for oxidized and reduced triphosphopyridine nucleotides; DPN and DPNH for oxidized and reduced diphosphopyridine nucleotides; GSSG and GSH for oxidized and reduced glutathione; ATP for adenosine triphosphate.
the reduction of GSSG was not influenced to a significant extent by the presence of phosphate or other salts, the DPN-linked system was markedly affected by these salts.

As shown in Table I, in the presence of DPN as cofactor, both phosphate and arsenate stimulated the reduction of glutathione, while marked inhibitions were observed with other salts, such as sodium chloride (potassium chloride, ammonium and sodium sulfate act similarly). A dependence of the reaction on the presence of a hydrogen donor system is also demonstrated in Table I. Since no comparable effects were observed when TPN was used as a hydrogen carrier, the possibility was considered that two separate enzymes, one DPN-linked, the other TPN-linked, are present in the crude yeast extract. However, the preparative procedure of the enzyme, to be described below, which resulted in a more than 250-fold purification over the crude extract, failed to separate the two activities.

Attempts were therefore made to elucidate the mechanism of the phosphate stimulation and sulfate inhibition. It was quite obvious that the latter did not inactivate the enzyme, since preparations stored in 50 per cent saturated ammonium sulfate solutions for several months were reactivated after removal of the salts by dialysis. A protective effect of phosphate against inactivation was also ruled out. It can be seen from Table II that there is only slight inactivation of an enzyme preparation incubated for 20 minutes at 37°, and there is no evidence for a protective action by addition of phosphate. It should also be pointed out that the stimulatory effect of phosphates is independent of the cation used, since

<table>
<thead>
<tr>
<th>Stimulation of Glutathione Reduction by Phosphate and Arsenate; Inhibitory Effect of Sodium Chloride</th>
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<tbody>
<tr>
<td>The complete system contained, per ml., 50 μmoles of glycylglycine, pH 7.6, 300 μmoles of ethyl alcohol, 50 γ of alcohol dehydrogenase, 1 μmole of DPN, 50 μmoles of potassium phosphate or arsenate, pH 7.8, 0.05 ml. of a partially purified yeast reductase preparation (Step 3), and 200 γ of oxidized glutathione. 50 μmoles of sodium chloride were added to demonstrate the inhibition. The centrifuge tubes were incubated for 10 minutes at 37°, and glutathione was determined as described under &quot;Materials and methods.&quot;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GSH, γ per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system with phosphate ..................................</td>
</tr>
<tr>
<td>Phosphate omitted ..................................................</td>
</tr>
<tr>
<td>Arsenate instead of phosphate .....................................</td>
</tr>
<tr>
<td>Alcohol omitted .....................................................</td>
</tr>
<tr>
<td>DPN omitted .........................................................</td>
</tr>
<tr>
<td>GSSG &quot; .........................................................</td>
</tr>
<tr>
<td>Complete with sodium chloride ....................................</td>
</tr>
</tbody>
</table>
potassium, ammonium phosphate, and sodium phosphate were all equally effective.

In the majority of experiments reported in this paper, alcohol, in the presence of alcohol dehydrogenase, was used as hydrogen donor to reduce DPN, and glucose-6-phosphate with glucose-6-phosphate dehydrogenase was used to reduce TPN. To demonstrate conclusively that the salt affected the glutathione reductase and not the reduction of the coenzymes, glucose dehydrogenase was selected as a hydrogen donor system, since it was capable of transferring hydrogens to DPN as well as TPN. It was found that either DPN or TPN can be used as hydrogen carrier from glucose to glutathione, and that only the DPN-linked system is inhibited by moderate concentration of sodium chloride (Table III).

**Table II**

**Stability of Glutathione Reductase in Presence and Absence of Phosphate**

The enzyme preparation used in this experiment was a crude dialyzed extract of dried yeast. 0.1 ml. of this extract was diluted to 0.5 ml. either with distilled water or with 0.1 M potassium phosphate buffer, pH 7.6. After incubation at 37° for 20 minutes the solution required for the DPN-linked test system (see Table I) was added, and GSH was measured after incubation for 10 minutes at 37°.

<table>
<thead>
<tr>
<th>Treatment of enzyme</th>
<th>Tested with phosphate</th>
<th>Tested without phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubated 20 min. at 37° without phosphate</td>
<td>125</td>
<td>38</td>
</tr>
<tr>
<td>&quot; 20 &quot; &quot; 37° with phosphate</td>
<td>125</td>
<td>45</td>
</tr>
<tr>
<td>Kept at 0°</td>
<td>138</td>
<td>45</td>
</tr>
</tbody>
</table>

**Purification of Yeast GSSG Reductase.** *Step 1—*Preparation of yeast extracts from 300 gm. of dried bakers’ yeast was carried out as described previously (7).

*Step 2—*Fractionation of the heated extract with acetone was carried out as for alcohol dehydrogenase (7), but the first precipitate after the addition of 0.5 volume of acetone was collected. This precipitate was extracted for 20 minutes with about 100 ml. of 0.01 M potassium phosphate, pH 7.4, and centrifuged. (These and the following steps were carried out at a temperature near 0° unless stated otherwise.)

*Step 3—*The supernatant solution was adjusted to pH 5.9 with 0.2 N acetic acid, and for each 100 ml. of solution 20 ml. of 95 per cent ethyl alcohol were slowly added at −3° (in an alcohol-dry ice bath) and centrifuged at −3° in a refrigerated centrifuge (10 minutes at 15,000 × g). The precipitate was dissolved in 30 ml. of 0.05 M potassium phosphate buffer, pH 7.6.
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Step 4—5 ml. of 2 per cent protamine sulfate (Nutritional Biochemicals Corporation) were added, and the precipitate was centrifuged. The supernatant solution was adjusted carefully to pH 5.5 with 0.1 N acetic acid. For each 10 ml. of solution, 2 ml. of 95 per cent ethyl alcohol were added, the solution being kept at -3° in an alcohol-dry ice bath. The mixture was centrifuged at -3° and the precipitate taken up in 10 ml. of H₂O.

Step 5—The pH of the solution was adjusted to 5.6, and 0.2 volume of calcium phosphate gel (20 mg. per ml.) was added. The mixture was centrifuged and the gel washed once with 5 ml. of H₂O, then eluted three times at room temperature with 3 ml. of 0.05 M potassium phosphate, pH 7.6.

**TABLE III**

Effect of Salts on Glutathione Reduction with Glucose Dehydrogenase and Glucose As Hydrogen Donor System

The system contained, per ml., 50 μmoles of tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.6, 250 μmoles of glucose, 400 γ of oxidized glutathione, 1.2 mg. of glucose dehydrogenase, 1 μmole of DPN or 0.1 μmole of TPN, 40 γ of a partially purified preparation of yeast GSSG reductase, 50 μmoles of potassium phosphate, pH 7.6 (where indicated), and 100 μmoles of sodium chloride (where indicated). The test was carried out as described in Table I.

<table>
<thead>
<tr>
<th>System</th>
<th>GSH, γ per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer and DPN</td>
<td>25</td>
</tr>
<tr>
<td>Phosphate buffer and DPN</td>
<td>114</td>
</tr>
<tr>
<td>&quot; &quot; DPN, and NaCl</td>
<td>38</td>
</tr>
<tr>
<td>Tris buffer and TPN</td>
<td>255</td>
</tr>
<tr>
<td>Phosphate buffer and TPN</td>
<td>250</td>
</tr>
<tr>
<td>&quot; &quot; TPN, and NaCl</td>
<td>230</td>
</tr>
</tbody>
</table>

Step 6—To the combined eluates, 2.8 gm. of solid dibasic ammonium phosphate were added per 10 ml. of solution, and the precipitate was centrifuged. To the supernatant solution, 1 additional gm. of ammonium phosphate per 10 ml. was slowly added, and the mixture was allowed to stand in an ice bath for 1 hour. The crystalline material was collected by centrifuging at 16,000 × g for 20 minutes. A typical protocol, showing data on the specific activity of the various fractions and the yield obtained, is given in Table IV.

It should be pointed out that some variation in the activity of crude extracts from different batches of yeast has been obtained, and it is essential to start with active extracts. Steps 1, 2, and 3 are quite reproducible, but in Step 4 the amount of protamine which can be used without encountering severe losses in activity varies from 3 to 7 ml. of a 2 per cent solution. It is essential, however, to remove most of the nucleic acid at this step, and
losses up to 25 per cent are acceptable. Variation in Step 5 may also be encountered, depending on the properties of the calcium phosphate gel. This, in fact, is the case in all procedures involving the use of aged gels. In case of difficulties in elution, 0.1 M phosphate buffers can be used.

Properties of Yeast Glutathione Reductase—Purified preparations of glutathione reductase from yeast are relatively stable if kept frozen at \(-20^\circ\). One preparation of 35 per cent purity (compared to the best preparations obtained to date) which was stored in 0.05 M phosphate buffer lost about half its activity during a period of 22 months storage in the deep freeze. However, in dilute solution rapid surface denaturation occurs. For the more sensitive spectrophotometric method with reduced TPN, the addition of serum albumin as a protecting agent is essential. For example, an en-

<table>
<thead>
<tr>
<th>Purification of Yeast GSSG Reductase</th>
<th>Units* per ml.</th>
<th>Specific activity*</th>
<th>Total units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>20,000</td>
<td>510</td>
<td>6,400,000</td>
</tr>
<tr>
<td>1st acetone ppt.</td>
<td>41,000</td>
<td>2,100</td>
<td>4,100,000</td>
</tr>
<tr>
<td>&quot; alcohol &quot;</td>
<td>96,000</td>
<td>6,000</td>
<td>2,880,000</td>
</tr>
<tr>
<td>2nd &quot; &quot;</td>
<td>210,000</td>
<td>30,000</td>
<td>2,100,000</td>
</tr>
<tr>
<td>Gel eluates</td>
<td>190,000</td>
<td>73,000</td>
<td>1,520,000</td>
</tr>
<tr>
<td>1st crystals</td>
<td>270,000</td>
<td>170,000</td>
<td>810,000</td>
</tr>
</tbody>
</table>

* The spectrophotometric test with TPNH was used for these assays. 1 unit of enzyme is defined as a change in \(\log I_0/I\) of 0.001 per minute under the above conditions. Specific activity is expressed as units of enzyme activity per mg. of protein.

zyme preparation diluted in potassium phosphate buffer, pH 7.6, was found to contain 5600 units per ml., whereas, when it was diluted in a solution containing buffer as well as 500 \(\gamma\) of bovine serum albumin per ml., it assayed at 18,000 units per ml. When 1 mg. of serum albumin was also added to the test system in the Beckman cell, the assay was 26,000 units per ml. The enzyme is rapidly inactivated when heated at temperatures above 60°. Although some losses occur during dialysis of the enzyme, no evidence for a coenzyme was obtained. A determination of the optimal concentration of the components of the test system for the reductase revealed the need for relatively high concentrations of DPN and also the inhibitory action of larger amounts of oxidized glutathione. This latter finding varied with different preparations of oxidized glutathione and may be partly due to an impurity in the preparations of oxidized glutathione. An inhibitory effect of GSSG was not noticed with TPN as coenzyme. The enzyme can be treated for 20 minutes with \(10^{-3}\) M sodium iodoacetate.
After brief dialysis it was found that little or no inactivation due to iodoacetate had occurred.

Purification of Liver Glutathione Reductase—Frozen beef liver, stored in the deep freeze at $-20^\circ$, was used as starting material. Acetone-dried powders were prepared as described previously (8). The extraction of the enzyme with water and fractionation with alcohol were the same as for aldehyde dehydrogenase, except that the first alcohol precipitate was collected and found to contain most of the reductase activity. The second step of purification consisted of isoelectric precipitations. First the fraction was adjusted to pH 6.5 to 6.6, and the precipitate was discarded. The supernatant solution was brought to pH 5.8 by the slow addition of 0.1 N acetic acid while the mixture was kept in an ice bath. After 20 minutes the precipitate was collected and dissolved with dilute alkali (final pH of 7.0).

<table>
<thead>
<tr>
<th>TABLE V</th>
<th>Purification of Liver GSSG Reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract (540 ml.)</td>
<td>15,000</td>
</tr>
<tr>
<td>1st alcohol ppt. (100 ml.)</td>
<td>37,000</td>
</tr>
<tr>
<td>pH 5.8, isoelectric ppt. (25 ml.)</td>
<td>92,000</td>
</tr>
</tbody>
</table>

* Units and specific activity as in Table IV.

The precipitation of the enzyme between pH 6.5 and 5.8 was repeated several times without undue losses of enzyme activity.

A typical protocol for the purification of the liver enzyme is given in Table V.

Properties of Liver Glutathione Reductase—The liver enzyme like the yeast enzyme reacts much more rapidly with TPN than with DPN. Glutathione reductase from liver can be precipitated at pH 5.8, while the yeast enzyme is completely soluble at this pH. The liver enzyme appears less sensitive to the inhibitory action of sodium chloride, but is markedly inhibited by 0.05 M sodium azide. The enzyme preparations with the highest specific activity obtained show the typical absorption spectrum of a protein, with a pronounced band at 410 m$, indicating the presence of a porphyrin. The possibility that this band might be due to an impurity cannot as yet be ruled out.

Coupling of Glutathione Reductase to Other Enzyme Systems—Glutathione reductase which is readily obtained from yeast in concentrated and purified solutions, as outlined above, is useful in following the course of other hydrogen transfer reactions. Its use for determination of the thiol hydrogen
transferase will be described in the following paper. The reductase can be conveniently used for the determination of dehydrogenase reactions which are not suitable for spectrophotometric tests. In the presence of excess glutathione reductase and limiting amounts of hydrogen donor system good proportionality between glucose dehydrogenase concentration and glutathione reduction has been obtained (Fig. 1).

**Fig. 1.** Determination of glucose dehydrogenase coupled to GSSG reductase. Experimental conditions essentially as in Table III, except that an excess of reductase (300 μ) and a limiting amount of glucose dehydrogenase were used.

**Fig. 2.** Spectrophotometric determination of GSSG reductase (liver).

**DISCUSSION**

It has been shown in the experiments described above that glutathione reductase from bakers' yeast and from beef liver react with both TPNH and DPNH. The peculiar properties of the enzyme in the reaction with DPNH help to explain why previous investigators have failed to observe this reaction. A stimulation by phosphate buffer, an inhibition by various other salts, and a low affinity for the DPNH are among the major factors which may have obscured the reaction. While at high nucleotide concentrations, with glucose as hydrogen donor, the rates with DPN and TPN
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are of the same order of magnitude, at low concentrations, as in spectrophotometric tests, over 100-fold differences in activity between DPNH and TPNH can be observed (see Fig. 2). Nevertheless, it is quite feasible to measure the reaction with DPNH spectrophotometrically and, under comparable conditions, very good agreement with the colorimetric test has been obtained.

The pronounced stimulation of DPN-linked reduction of glutathione by phosphate has been investigated by several approaches. The possibility of a participation of phosphate in the DPN-linked reaction was entertained in terms of a phosphorolytic cleavage of the S–S bond. Experiments were designed to explore this possibility by using P32-labeled inorganic phosphate with ATP added to the reaction mixture. With partially purified preparations of glutathione reductase it was found that P32 was rapidly incorporated into ATP, which was separated on a Dowex column, according to Cohn (9). Residual fermentation in the partially purified preparation may have been responsible, since highly purified preparations of glutathione reductase did not catalyze the incorporation of P32 into ATP. On the other hand, the fact that incorporation of P32 into ATP was stimulated by oxidized glutathione and by alcohol lent support to the thesis that the incorporation of P32 was at least partly due to a mechanism other than fermentation.

The possible rôle of glutathione reductase has been discussed in previous publications (4–10). The usefulness of glutathione reductase for the detection of nucleotide-linked dehydrogenases in systems containing active reductase activity has been pointed out by Vennesland (10). The addition of an excess of purified glutathione reductase has been shown in this paper to facilitate the quantitative aspect of this method. However, it should be pointed out that side reactions leading to disappearance of reduced glutathione have been encountered in crude extracts and precautionary procedures, e.g. anaerobic conditions, may be required with some preparations. Glutathione reductase, linked to "malic" enzyme, has been used for a very sensitive assay of TPN, and has also been used in the Hill reaction (cf. (10)).

Finally, it may be mentioned that glutathione reductase can serve as a specific reagent for the quantitative estimation of oxidized glutathione (11).

Materials and Methods

Substrates and Coenzymes—GSH and GSSG were obtained commercially from the Schwarz Laboratories, Inc. Small batches of GSSG were also prepared by oxidation of reduced GSH either with hydrogen peroxide or by aeration at alkaline pH in the presence of catalytic amounts of iron salts. TPN "80" was obtained from the Sigma Chemical Company; DPN
was prepared in this laboratory and was assayed enzymatically to be 67 per cent pure. Reduced DPN was prepared according to Ohlmeyer (12) and reduced TPN according to Kaplan et al. (13). Glucose-6-phosphate was prepared according to Levene and Raymond (14). Tris(hydroxymethyl)aminomethane was obtained from the Sigma Chemical Company.

Auxiliary Enzymes—Alcohol dehydrogenase was prepared from bakers’ yeast (7), glucose-6-phosphate dehydrogenase from brewers’ yeast (15) and stored in solution in the deep freeze. Glucose dehydrogenase from beef liver (16) was kindly supplied by Dr. H. J. Strecker. Glyoxalase I was prepared as described previously (17).

Colorimetric Determination of Reduced Glutathione—The formation of reduced glutathione was measured by the nitroprusside test essentially as described by Grunert and Phillips (18), except that the final volume was reduced to 2.5 ml. With active preparations of glutathione reductase there was no need to deproteinize the samples, and the DPN-catalyzed reaction was effectively stopped by saturation with sodium chloride. A tube to which all reagents except DPN or oxidized glutathione were added served as control.

Enzymatic Assay of Glutathione—Reduced glutathione was determined by
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the glyoxalase test as described previously (17) or by a spectrophotometric assay with glyoxalase I.

Spectrophotometric Test for Glutathione Reductase—In a final volume of 1 ml. (micro cells for the Beckman DU spectrophotometer) 50 μmoles of potassium phosphate, pH 7.6, 0.1 μmole of reduced TPN, 1 mg. of bovine serum albumin, glutathione reductase (diluted in 0.05 m phosphate buffer containing 500 γ of bovine serum albumin per ml.) and 3.3 μmoles of oxidized glutathione were mixed. The reoxidation of reduced TPN was followed at 340 μm in a Beckman spectrophotometer at 30 second intervals. Satisfactory proportionality was obtained between enzyme concentration and rate of TPNH oxidation (Fig. 2).

Colorimetric Test for Glutathione Reductase—The test system contained, per ml., 300 μmoles of ethyl alcohol, 50 μmoles of potassium phosphate, pH 7.6, 1 μmole of DPN, about 100 γ of alcohol dehydrogenase, and 300 γ of oxidized glutathione. The mixture was warmed to 37°; after addition of the enzyme to be tested, it was kept at that temperature for 10 minutes and then assayed for reduced glutathione colorimetrically. Proportionality between enzyme concentration and colorimetric units was not quite as satisfactory as with the spectrophotometric test, but suitable within a fairly limited range of enzyme concentration (Fig. 3).

Protein Determinations—Protein concentrations were measured spectrophotometrically at 280 μm. Corrections for nucleic acids were made according to Warburg and Christian (19). With crude preparations the quantitative biuret test was used (20).

SUMMARY

1. Glutathione reductase has been purified from bakers’ yeast and beef liver, and its properties are reported.
2. The purified enzymes can catalyze the hydrogen transfer from both DPNH and TPNH, but more rapidly with the latter.
3. The DPN-linked transfer of the yeast enzyme is very sensitive to the inhibitory action of salts such as sodium or potassium chloride and is stimulated by phosphates. The TPN-linked reaction is not affected by these salts.
4. The usefulness of a purified preparation of glutathione reductase for enzyme studies and glutathione determination is discussed.

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