Purification of Thioredoxin, Thioredoxin Reductase, and Glutathione Reductase by Affinity Chromatography*

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A scheme is described for the large scale purification of thioredoxin, thioredoxin reductase, and glutathione reductase. The scheme is based on an initial separation of thioredoxin from the two reductases by affinity chromatography on agarose-bound N^6-(6-aminohexyl)-adenosine 2',5'-bisphosphate (agarose-2',5'-ADP). The two reductases were then separated by hydrophobic chromatography and purified separately to homogeneity. Thioredoxin was purified to homogeneity by immunoadsorption to agarose containing immobilized goat anti-thioredoxin. Overall yields for thioredoxin, thioredoxin reductase, and glutathione reductase exceeded 80% in each case. Both reductases exhibit an absorption band at approximately 320 nm which appears due to a residual amount of tightly bound NADP. Presence of this absorption band has no apparent effect on the specific activity of either enzyme.

In Escherichia coli the reduction of ribonucleotides is carried out by the enzyme ribonucleoside diphosphate reductase. This reduction reaction may be coupled in vitro to the oxidation of NADPH via the small disulfide protein, thioredoxin, and thioredoxin reductase (1). The facility that the thioredoxin system has for performing this reaction together with the finding of analogous thioredoxins in various cell types led to the acceptance of this biosynthetic pathway as the in vivo mode of deoxyribonucleotide synthesis. However, two recent findings question the exclusiveness of thioredoxin in the reduction of ribonucleotides in vivo. First, thioredoxin is implicated in several other biosynthetic reactions including the reduction of disulfides in proteins and simple compounds (2), the reduction of sulfoxides to thioethers (3), the reduction of sulfate to sulfite (3, 4), and its role as an essential subunit for phage T7 DNA polymerase (5). Second, bacterial mutants lacking thioredoxin activity grow normally (6). Moreover, in mutants lacking thioredoxin, a second protein exists that couples the NADPH oxidation to ribonucleotide reduction (7). However, this protein, "glutaredoxin," is cyclically reduced by glutathione, which in turn is reduced by glutathione reductase. Thus, there appear to be two parallel but mutually exclusive pathways for reducing ribonucleotides through either the thioredoxin or the glutathione systems.

Interest in studying the supportive roles of these two NADPH-dependent reductases in the reduction of ribonucleotides led us to devise a convenient means for obtaining each enzyme in high yields. Consequently, methodologies were developed involving an initial copurification of the two NADP(H)-binding proteins by affinity chromatography on agarose-2',5'-ADP, followed by an efficient separation of the two reductases by hydrophobic chromatography on agarose/hexylamine. Each reductase is then purified (separately) to homogeneity by an additional column step yielding an overall purification yield of approximately 80% for either protein. Thioredoxin conveniently separates from its cognate reductase at the first purification step; the thioredoxin fraction is then readily purified to 80% of homogeneity on a preparative scale immunoadsorption column.

RESULTS

Purification of Thioredoxin Reductase and Glutathione Reductase

* Portions of this paper (including Figs. 2 through 5, Tables I and II, and the references) are presented in miniprint following the References. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document #76M-925, cite author(s), and include a check or money order for $2.25 per set of photocopies.

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Affinity Purification: Thioredoxin and Glutathione Reductases

Fig. 1. Agarose/hexylamine chromatography of glutathione reductase and thioredoxin reductase after DE52 purification. Samples of glutathione reductase and thioredoxin reductase each purified according to Williams et al. (8) through the DE52 column step were purified first on agarose-2',5'-ADP, then applied to separate agarose/hexylamine columns. A, the glutathione reductase fraction contained \( A_{636} = 7.8 \) and 9970 units of glutathione reductase. B, thioredoxin reductase pool contained \( A_{570} = 3.2 \) and 793 units of thioredoxin reductase. Elution was effected with a 60-mI linear gradient of NaCl 0 to 1 M in 10 mM phosphate buffer (pH 7.2), 10 mM EDTA. One milliliter fractions were collected and analyzed for \( A_{340} \), glutathione reductase (○—△), and thioredoxin reductase (○—○). The salt gradient was monitored by conductivity (×—×). C, densitometer profiles of SDS-polyacrylamide gels for each enzyme fraction after agarose-hexylamine chromatography. Sample A, glutathione reductase from A; Sample B, glutathione reductase from B; Sample C, thioredoxin reductase from B.

**Fractionation on Agarose-2',5'-ADP—**Preliminary experiments with agarose-2',5'-ADP showed that both reductases were effectively bound and subsequently eluted with modest concentrations of NADPH (Table I). Optimal conditions for purifying either thioredoxin reductase or glutathione reductase on this ADP affinity column were determined with fractions of each protein purified through the DE52 column step of Williams et al. (8) Each fraction was applied to separate agarose/ADP columns (1 ml) in low ionic strength buffer (10 mM phosphate) followed by 3-column volumes of column buffer containing 200 mM NaCl. For practical purposes, column capacity was determined as the amount of enzyme retained when 10% of the applied activity appeared in the wash fractions. For the glutathione reductase fraction 600 units and 20 mg of protein (total) were retained per ml of gel.

Elution of activity was most effective using a 2.0-mI "pulse" of 1 mM NADPH (in 10 mM phosphate, pH 7.6, 3 mM EDTA) followed by 1-column volume of buffer without coenzyme. Glutathione reductase was eluted quantitatively, and subsequent chromatography of this fraction on agarose/hexylamine (Fig. 1A) revealed a single protein peak, corresponding to the peak of glutathione reductase activity. Polyacrylamide gel (SDS\(^+\)) electrophoresis of the fraction after agarose/hexylamine revealed a major band constituting 75% of the total protein and numerous minor components (Fig 1C).

The comparative thioredoxin reductase fraction taken from the Williams DE52 column was retained to the extent of 570 units/ml of agarose-2',5'-ADP. Fifteen milligrams of protein were bound per ml of gel. Agarose/hexylamine chromatography of the thioredoxin reductase fraction obtained from the ADP affinity column produced two major protein peaks corresponding to thioredoxin reductase and contaminating glutathione reductase (Fig. 1B). SDS-gel electrophoresis of the thioredoxin reductase pool revealed two species present in approximately equal amounts with molecular weights of 3.4 \( \times \) 104 (thioredoxin reductase) and 4.6 \( \times \) 104 (Fig. 1C).

**Characterization of the Homogeneous Proteins—**Data characterizing the purified thioredoxin reductase and glutathione reductase are present in Table III. The thioredoxin reductase fraction after DE52 chromatography (Fig. 3) had a uniform specific activity upon chromatography on Sephadex G-100 and eluted at a molecular weight corresponding to 6.8 \( \times \) 104. The specific activity of the DE52 enzyme was determined as 1290 units/A\(_{340}\) (or 1810 units/mg using the standard DTNB coupled assay). Adaptation of this same assay to measure NADPH oxidation (with substrate amounts of thioredoxin) showed a specific activity of 67.0 \( \mu \)mol of NADPH oxidized/min/mg, comparable to the best preparations reported by others (8, 9).

The glutathione reductase pool after agarose/hexylamine chromatography was substantially contaminated with several protein species, but further fractionation on DE52 and Bio-Gel A-0.5 yielded a contaminant-free protein (Fig. 5). Polyacrylamide gels overloaded with 60 \( \mu \)g of the protein purified through the Bio-Gel step revealed only one staining species at 5.2 \( \times \) 104 daltons. The molecular weight of glutathione reductase characterized by chromatography using Bio-Gel A-0.5 was calculated as 1.95 \( \times \) 105 (Fig. 4B). The specific activity of the Bio-Gel fraction was 2700 units/A\(_{340}\) using the standard assay (or 510 \( \mu \)mol of NADPH oxidized/min/mg). This value is comparable to that reported for the protein purified by Williams and Arscott (10).

The absorption spectra for glutathione reductase and thioredoxin reductase (Fig. 6) revealed both proteins as typical flavoproteins with an absorption peak at about 450 nm characteristic of the oxidized flavin. In contrast to the spectra published for either reductase, however, each protein purified by the affinity procedures here revealed an absorption band centered at approximately 320 to 330 nm (11, 12). For example, the A\(_{320}\)/A\(_{280}\) ratio for glutathione reductase purified by conventional means was 4.0 (A\(_{340}/A_{280} = 4.5\) for FAD); different preparations of the affinity purified enzyme had a spectral ratio ranging from 2 to 1. The 320 nm chromophore is apparently intro-

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2 The abbreviations used are: SDS, sodium dodecyl sulfate; DTNB, 5,5-dithiobis(2-nitrobenzoic acid).
TABLE III

<table>
<thead>
<tr>
<th>Property</th>
<th>Glutathione reductase</th>
<th>Thioredoxin reductase</th>
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</thead>
<tbody>
<tr>
<td>Molecular weight (Rin-Gel A-0.5m chromatography)</td>
<td>$1.05 \times 10^4$</td>
<td>$6.8 \times 10^4$</td>
</tr>
<tr>
<td>Molecular weight*</td>
<td>$5.2 \times 10^4$</td>
<td>$3.4 \times 10^4$</td>
</tr>
<tr>
<td>$E_{280}, 1 \text{ mg/ml}$</td>
<td>1.54</td>
<td>1.39</td>
</tr>
<tr>
<td>$A_{325}/A_{260}$</td>
<td>8.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Specific activity $\Delta A_{340}$/min/mg</td>
<td>1800</td>
<td>505</td>
</tr>
<tr>
<td>Micromoles of NADPH oxidized/min/mg</td>
<td>67.0</td>
<td>505</td>
</tr>
</tbody>
</table>

* Molecular weights of the polypeptide chains were determined by polyacrylamide gel electrophoresis in the presence of 0.1 M mercaptoethanol and 1% SDS.

Enzyme activities are expressed as units per mg of protein based on the determined extinction coefficients. Thioredoxin reductase activity is expressed either as the reduction of DTNB in the coupled assay used during purification or as the consumption of NADPH. In the latter case thioredoxin was used in substrate amounts in the standard assay mixture lacking DTNB and monitoring the oxidation of NADPH by the reduction of absorbance at 340 nm.

**DISCUSSION**

Earlier purification schemes for thioredoxin, thioredoxin reductase, and glutathione reductase involved numerous column chromatography steps, and only one scheme (8) attempted purification of all three proteins from a single extract. Yields of each protein obtained by these purification schemes were varied and uniformly low for thioredoxin (25% (13); 50% (8)), thioredoxin reductase (25% (1); 29% (8)), and glutathione reductase (28% (8)). Purification of thioredoxin by immunoadsorption (14) allowed for an efficient and expedient purification, but for only one of the desired three proteins. All described purification schemes for the thioredoxin and glutathione reductases involve a heat denaturation step (7°, 5 min (8); 65°, 5 min (2)), and in one case an acid denaturation step (2) is also involved. Affinity purification of thioredoxin and glutathione reductases obviated the harsh treatment used in the conventional purification schemes and provided an expedient means for obtaining both proteins in high yield.
Several factors influenced the choice of when to use the affinity column in the overall purification scheme. When affinity chromatography is used as the first step, each reductase is purified 300-fold. The alternative approach of affinity chromatography after several initial purification steps has the virtue of a much greater column capacity. Thus, glutathione reductase and thioredoxin reductase each purified through the DEAE-step of Williams et al. (8), bind to the extent of 600 and 570 units/ml of gel, respectively. In contrast, only 116 and 26 units/ml gel of each enzyme bind using a crude extract. This disparity probably results from the presence in crude extracts of several NADP(H)-binding proteins competing with the desired proteins for the immobilized nucleotide. Use of 2',5'-ADP as a "general ligand" for purification of NADP(H) proteins has in fact been proposed by Brodelius et al. (15). Our desire for a purification scheme lacking drastic conditions such as heat or acid denaturation made it preferable to use an affinity column as the initial purification step.

Purity of the enzymes obtained by the preparative scheme was judged by criteria of polyacrylamide gel electrophoresis in SDS (Fig. 5), constancy of specific activity through pooled column peaks (Figs. 3 and 4B), and enzymatic specific activities. The specific activity of purified thioredoxin reductase was 1290 units/A\textsubscript{280} using the DTNB-coupled reaction and 67 \mu mol of NADPH/min/mg assayed with substrate levels of thioredoxin. These values compare well with the value of 1020 units/A\textsubscript{280} reported by Williams et al. (8) and 44 \mu mol of NADPH/min/mg reported by Thelander (9).

Final preparations of glutathione reductase showed an activity of 214 \mu mol/min/A\textsubscript{280}, in good agreement with the value of 210 \mu mol/min/A\textsubscript{280} (enzyme purified to approximately 90%) described by Williams and Arscott (10).

Both spectral and isotope studies show that the anomalous absorption band in the range of 320 to 340 nm was introduced as a result of the affinity procedure. The structural nature of nucleotide chromophore was not defined, but it could represent an NADP-enzyme intermediate such as that described for the flavoprotein cytochrome b\textsubscript{5} reductase (Strittmatter (11); Williams (12)). This latter enzyme has a stable absorption band at 317 nm attributed to the partially reduced enzyme-NADP complex. It is not certain as to whether this chromophore is a normal component of these enzymes, however, presence of this absorption had no apparent detrimental effect on enzymatic activity. Although the presence of NADP on either glutathione or thioredoxin reductase has not previously been documented, the presence of NAD on purified dehydrogenase is well documented. For example, affinity-purified lactate dehydrogenases contain tightly bound NAD which is removable only by drastic procedures, using charcoal absorption or displacement with a tighter binding analog (Wieland et al. (13); Jago et al. (14)).

Existence of a relatively stable NADP-enzyme complex should aid considerably in elucidating the catalytic mechanism of both reductases. The availability of large quantities of each protein now permits such a detailed mechanistic approach.
Affinity Purification: Thioredoxin and Glutathione Reductases

Materials and Methods

Chemicals and reagents were obtained from the following commercial sources: Thioredoxin (from Escherichia coli) and Glutathione reductase (from horse liver) from Sigma Chemical Co. (St. Louis, MO); thioredoxin and glutathione reductase specific antibodies were raised in rabbits by Abaxis (Union City, CA), 2-thioglycerol from MP Biomedical (Solon, OH); NADPH from Sigma Chemical Co.; dithionite from Aldrich Chemical Co. (Milwaukee, WI); 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) from Dojindo Laboratories (Kumamoto, Japan); and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG from Jackson ImmunoResearch Laboratories (West Grove, PA).

Affinity Chromatography

The thioredoxin and glutathione reductase affinity matrices were prepared by binding 1 mg of recombinant thioredoxin and 1 mg of recombinant glutathione reductase to 1 ml of Sepharose 4B with 1 M ethanolamine-HCl buffer pH 8.5. The matrices were then washed extensively with buffer containing 0.5 M NaCl to remove any unbound reagents. The matrices were then packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol.

Procedures for Preparation of Affinity-Chromatography Columns

The thioredoxin- and glutathione reductase affinity matrices were packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M NaCl to remove any unbound reagents. The column was then washed with buffer containing 10 mM potassium phosphate buffer pH 7.4, 10 mM 2-thioglycerol, and 10 mM dithiothreitol.

Protein Binding

Affinity Purification of Thioredoxin

The thioredoxin- and glutathione reductase affinity matrices were packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M NaCl to remove any unbound reagents. The column was then washed with buffer containing 10 mM potassium phosphate buffer pH 7.4, 10 mM 2-thioglycerol, and 10 mM dithiothreitol.

Protein Binding

Affinity Purification of Glutathione Reductase

The glutathione reductase affinity matrix was packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M NaCl to remove any unbound reagents. The column was then washed with buffer containing 10 mM potassium phosphate buffer pH 7.4, 10 mM 2-thioglycerol, and 10 mM dithiothreitol.

Protein Binding

Affinity Purification of Glutathione Reductase

The glutathione reductase affinity matrix was packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M NaCl to remove any unbound reagents. The column was then washed with buffer containing 10 mM potassium phosphate buffer pH 7.4, 10 mM 2-thioglycerol, and 10 mM dithiothreitol.

Protein Binding

Affinity Purification of Glutathione Reductase

The glutathione reductase affinity matrix was packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M NaCl to remove any unbound reagents. The column was then washed with buffer containing 10 mM potassium phosphate buffer pH 7.4, 10 mM 2-thioglycerol, and 10 mM dithiothreitol.

Protein Binding

Affinity Purification of Glutathione Reductase

The glutathione reductase affinity matrix was packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M NaCl to remove any unbound reagents. The column was then washed with buffer containing 10 mM potassium phosphate buffer pH 7.4, 10 mM 2-thioglycerol, and 10 mM dithiothreitol.

Protein Binding

Affinity Purification of Glutathione Reductase

The glutathione reductase affinity matrix was packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M NaCl to remove any unbound reagents. The column was then washed with buffer containing 10 mM potassium phosphate buffer pH 7.4, 10 mM 2-thioglycerol, and 10 mM dithiothreitol.

Protein Binding

Affinity Purification of Glutathione Reductase

The glutathione reductase affinity matrix was packed into a column (6 × 1 cm) and equilibrated with 10 mM potassium phosphate buffer pH 7.4 and 10 mM 2-thioglycerol. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.5 M NaCl to remove any unbound reagents. The column was then washed with buffer containing 10 mM potassium phosphate buffer pH 7.4, 10 mM 2-thioglycerol, and 10 mM dithiothreitol.

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Affinity Purification of Glutathione Reductase

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Affinity Purification: Thioredoxin and Glutathione Reductases

The thioredoxin and glutathione reductases were purified by affinity chromatography on thioredoxin-Sepharose and glutathione-Sepharose columns, respectively. The purification was carried out under aerobic conditions at 4°C, and the enzymes were eluted with 10 mM reduced glutathione.

**Table I**

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<tr>
<th>Purity</th>
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<th>Protein</th>
<th>Glutathione Reductase</th>
<th>Thioredoxin Reductase</th>
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</thead>
<tbody>
<tr>
<td>native</td>
<td>1.0 mL</td>
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<td>1.0 mg</td>
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<td>affinity</td>
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<td>affinity Sepharose</td>
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</table>

**Figure 1**

*Fig. 1.* Gel filtration of thioredoxin redctase.

**Figure 2**

*Fig. 2.* Gel filtration of glutathione reductase.

**Figure 3**

*Fig. 3.* Polyacrylamide gel electrophoresis of thioredoxin from different purification stages.

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**Table II**

<table>
<thead>
<tr>
<th>Purity</th>
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<th>Protein</th>
<th>Glutathione Reductase</th>
<th>Thioredoxin Reductase</th>
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<td>native</td>
<td>1.0 mL</td>
<td>1.0 mg</td>
<td>1.0 mg</td>
<td>1.0 mg</td>
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<tr>
<td>affinity</td>
<td>0.5 mL</td>
<td>0.5 mg</td>
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</tr>
<tr>
<td>affinity Sepharose</td>
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<td>0.2 mg</td>
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</tr>
</tbody>
</table>

**Figure 4**

*Fig. 4.* Glutathione reductase activity.

**Figure 5**

*Fig. 5.* Thioredoxin activity.

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**References**

Purification of thioredoxin, thioredoxin reductase, and glutathione reductase by affinity chromatography.
V P Pigiet and R R Conley


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