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-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 sulfides to thioethers (3), the reduction of sulfate to sulfite (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 co-purification 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 immunosaffinity 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.
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 step were purified first on agarose-2',5'-ADP, then applied to separate agarose/hexylamine columns. A., the glutathione reductase fraction contained A$_{340}$ = 7.8 and 9870 units of glutathione reductase. B., the thioredoxin reductase pool contained A$_{340}$ = 3.2 and 793 units of thioredoxin reductase. Elution was effected with a 60-ml 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 ( ).

**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 x 10^4. 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 μmol 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 μg of the protein purified through the Bio-Gel step revealed only one staining species at 5.2 x 10^4 daltons. The molecular weight of glutathione reductase chromatography using Bio-Gel A-0.5 was calculated as 1.05 x 10^5 (Fig. 4B). The specific activity of the Bio-Gel fraction was 2700 units/A$_{340}$ using the standard assay (or 510 pmol 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 ~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$_{340}$/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-
Table III

<table>
<thead>
<tr>
<th>Property</th>
<th>Glutathione reductase</th>
<th>Thioredoxin reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (Bio-Gel A-0.5m chromatography)</td>
<td>1.05 × 10^4</td>
<td>6.8 × 10^4</td>
</tr>
<tr>
<td>Molecular weight*</td>
<td>5.2 × 10^4</td>
<td>3.4 × 10^4</td>
</tr>
<tr>
<td>E290, 1 mg/ml</td>
<td>1.54</td>
<td>1.39</td>
</tr>
<tr>
<td>A340/A280</td>
<td>8.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Specific activity* A340/min/mg</td>
<td>1800</td>
<td>1800</td>
</tr>
<tr>
<td>Micromoles of NADPH oxidized/min/mg</td>
<td>505</td>
<td>67.0</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 assay.

Fig. 6. Absorption spectra of glutathione reductase and thioredoxin reductase. Spectra were obtained on a Cary 14 recording spectrophotometer at room temperature. Data for thioredoxin reductase (A) and glutathione reductase (B) are reported in terms of extinction coefficient A580/absorbance determined as described under "Materials and Methods."
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/A₂₈₀ using the DTNB-coupled reaction and 67 μmol of NADPH/min/mg assayed with substrate levels of thioredoxin. These values compare well with the value of 1020 units/A₂₈₀ reported by Williams et al. (8) and 44 μmol of NADPH/min/mg reported by Thelander (9).

Final preparations of glutathione reductase showed an activity of 214 μmol/min/A₂₈₀, in good agreement with the value of 210 μmol/min/A₂₈₀ (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 b₅ 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

Supplementary methods on Affinity Purification of Thioredoxins, Thioredoxin A, and Glutathione Reductase Activities by Affinity Chromatography by Haimin P. Peteh and Robert R. Hilton

MATERIALS AND METHODS

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Chemicals and reagents were obtained from the following commercial sources: Recombinant human thioredoxin (GeneTech Corp., Shanghai, China) and glutathione reductase (Sigma Chemical Co., St. Louis, MO). Thioredoxin and glutathione reductase were purified as previously described (16). Thioredoxin was purified further by affinity chromatography on glutathione agarose (Pharmacia, Piscataway, NJ). Glutathione reductase was purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ). Glutathione reductase activity was measured as previously described (16). Thioredoxin and glutathione reductase were co-purified on a gel capillary electrophoresis (GCE) assay using a gel capillary electrophoresis system (Pharmacia, Piscataway, NJ).

pH calculations were performed using the following equations:

\[ \text{pH} = -\log_{10}(\text{[H}^+\text{])} \]

where [H+] is the concentration of hydrogen ions.

The pH was adjusted to 7.4 using ice-cold 0.1M potassium phosphate buffer.

Affinity chromatography was performed as follows:

1. A 1-mL column of glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ) was equilibrated with 20 mL of 20 mM potassium phosphate buffer, pH 7.4.
2. Thioredoxin and glutathione reductase were loaded onto the column and washed with 20 mL of 20 mM potassium phosphate buffer, pH 7.4.
3. Fractions were collected and assayed for thioredoxin and glutathione reductase activity.

RESULTS

Affinity chromatography was performed using 10 mL of glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ) that had been equilibrated with 20 mL of 20 mM potassium phosphate buffer, pH 7.4. Thioredoxin and glutathione reductase were loaded onto the column and washed with 20 mL of 20 mM potassium phosphate buffer, pH 7.4. Fractions were collected and assayed for thioredoxin and glutathione reductase activity.

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Affinity Purification: Thioredoxin and Glutathione Reductases

TABLE II

<table>
<thead>
<tr>
<th>Purification</th>
<th>Protein Content</th>
<th>Glutathione Reductase Activity</th>
<th>Glutathione Reductase Activity (kU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st chromatography</td>
<td>1.3 mg</td>
<td>1.8 x 10^-4</td>
<td>1.8 x 10^-4</td>
</tr>
<tr>
<td>2nd chromatography</td>
<td>0.5 mg</td>
<td>2.1 x 10^-4</td>
<td>2.1 x 10^-4</td>
</tr>
</tbody>
</table>

Fig. 3

Affinity chromatography of thioredoxin reductase

The thioredoxin reductase was purified by affinity chromatography and dialyzed as described in the Experimental Procedures. The purified enzyme was assayed for its ability to reduce dithionite and to oxidize NADPH. The results are shown in Table II.

Fig. 4

Affinity chromatography of glutathione reductase

The glutathione reductase was purified by affinity chromatography and dialyzed as described in the Experimental Procedures. The purified enzyme was assayed for its ability to reduce glutathione disulfide and to oxidize NADPH. The results are shown in Table II.

REFERENCES


Fig. 5

Polyacrylamide gel electrophoresis of thioredoxin from different purification stages

Samples derived from Fig. 1 (lane 1) were subjected to polyacrylamide gel electrophoresis as described in the Experimental Procedures. The results are shown in Fig. 5.

Fig. 6

Polyacrylamide gel electrophoresis of glutathione reductase from different purification stages

Samples derived from Fig. 1 (lane 2) were subjected to polyacrylamide gel electrophoresis as described in the Experimental Procedures. The results are shown in Fig. 6.
Purification of thioredoxin, thioredoxin reductase, and glutathione reductase by affinity chromatography.
V P Pigiet and R R Conley