Bovine Thioredoxin System

PURIFICATION OF THIOREDOXIN REDUCTASE FROM CALF LIVER AND THYMUS AND STUDIES OF ITS FUNCTION IN DISULFIDE REDUCTION*

(Received for publication, September 28, 1976, and in revised form, February 9, 1977)

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Thioredoxin reductase (EC 1.6.4.5), which catalyzes the reduction of the disulfide bridge in thioredoxin by NADPH, was purified from calf liver and thymus. Preparation methods, involving chromatography on DEAE-cellulose, TEAE-cellulose, and Sephadex G-200 or G-100 were used to purify the calf liver enzyme 1100-fold and the thymus enzyme 2800-fold. The enzyme was shown to catalyze an NADPH-dependent reduction of 5,5'-dithiobis(2-nitrobenzoic acid) which could be used to develop a simple and rapid assay in addition to a specific calf liver thioredoxin-dependent reduction of disulfide bonds in bovine insulin.

The purified enzyme, which was inhibited by 0.1 mM arsenite, showed a wider substrate specificity than the corresponding enzyme from Escherichia coli and rapidly reduced E. coli thioredoxin, yeast thioredoxin, and 5,5'-dithiobis(2-nitrobenzoic acid). Phage T7 thioredoxin was slowly reduced. The apparent $k_m$ values for 5,5'-dithiobis(2-nitrobenzoic acid) and calf liver thioredoxin were 1.5 mM and 5 $\mu$M, respectively. In vitro oxidized preparations of calf liver thioredoxin were shown to contain high molecular weight mixed disulfide aggregates that were reduced by the enzyme with kinetics which supported a process of autoactivation. This may be of importance as a control mechanism for the activity of the bovine thioredoxin system.

Reduction of disulfide bonds in insulin or 1-cystine by NADPH and thioredoxin reductase was absolutely dependent upon the presence of thioredoxin as intermediate electron carrier. With the coupled system, fast reduction of insulin was obtained with as little as $3 \times 10^{-7}$ mM calf liver thioredoxin. By a number of criteria the bovine thioredoxin reductase shows a high specificity for the reduction of the disulfide bond of thioredoxin and will not react with protein disulfides, DTNB or the homologous thioredoxin-S, from yeast (5, 6).

Reduction of disulfide bonds in proteins will occur rapidly by thioredoxin-(SH)$_2$ (1, 2) according to Reaction 2 shown below:

$$\text{Thioredoxin-(SH)$_2$ + protein-S$_2$} \rightarrow \text{thioredoxin-(SH)$_2$ + protein-(SH)$_2$}$$

The equilibrium of this reaction will normally be shifted to the right due to the low oxidation-reduction potential of the thioredoxin-(SH)$_2$/thioredoxin-S, couple ($-0.26$ V at pH 7 and $25^\circ$C) (7). In the presence of thioredoxin reductase and an excess of NADPH, a fast reduction of disulfide bonds by NADPH will take place through a combination of Reactions 1 and 2. Under these conditions the low oxidation-reduction potential of NADPH ($-0.31$ V at pH 7 and $25^\circ$C) (8) drives the reduction of the disulfide bonds in a protein like insulin to completion.

Thioredoxin and thioredoxin reductase in mammalian cells were originally identified by their capacity to be the hydrogen donor system for E. coli ribonucleotide reductase in the presence of NADPH. Partially purified preparations of thioredoxin reductase from rat Novikoff hepatoma and regenerating rat liver have been obtained (9, 10).

1 The abbreviations used are: DNMB, 5,5'-dithiobis(2-nitrobenzoic acid); TNB, thionitrobenzoate; thioredoxin-S, and thioredoxin-(SH)$_2$.

* This investigation was supported by grants from the Swedish Medical Research Council, Project 13X-3529 and 13P-4292; Magnus Bergvalla Stiftelse and Harald Jeannons Stiftelse.

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The enzyme in having a wider substrate specificity and catalyzed protein disulfide bonds, however, required the presence of thiolation. The enzyme was different from the corresponding E. coli thioredoxin reductase from calf liver and thymus will be described based on its capacity to catalyze NADPH-dependent disulfide reduction by thioredoxin. By several criteria this bovine thioredoxin reductase (Fraction V), determined by Method 1 were added, and the mixtures were incubated for 20 min at 37°. The reaction was stopped by addition of 0.50 ml of 6 M guanidine hydrochloride, 50 mM Tris/Cl, pH 8.0, containing 10 mM DTNB and the absorbance at 412 nm was determined against a blank without enzyme. For further details, see miniprint supplement.

In a previous paper (11) we described the isolation of homogenous thioredoxin from calf liver. The protein has a molecular weight of 12,000 and the amino acid sequence of the active center disulfide bridge was found to be the same as in E. coli thioredoxin.

In this paper the purification and assay of thioredoxin reductase from calf liver and thymus will be described based on its capacity to catalyze NADPH-dependent disulfide reduction. The enzyme was different from the corresponding E. coli enzyme in having a wider substrate specificity and catalyzed an NADPH-dependent reduction of the disulfide bond in DTNB which could be used as a simple assay. Reduction of protein disulfide bonds, however, required the presence of thioredoxin. By several criteria this bovine thioredoxin reductase seems to be identical with the enzyme NADPH-protein disulfide reductase (EC 1.6.6.4) (12) or protein disulfide reductase previously identified in rat liver (13, 14) and shown to increase during the proliferation of murine tumors (15).

RESULTS

Assay of Thioredoxin Reductase from Calf

A rapid and simple spectrophotometric assay of thioredoxin reductase from E. coli or yeast utilizes the reduction of the homologous thioredoxin-S-S and its reoxidation by DTNB. This assay could not be applied to calf liver thioredoxin reductase because calf liver thioredoxin contains additional sulfhydryl groups (11), which apparently are modified by DTNB leading to inactivation. Furthermore, the calf liver enzyme reduced DTNB directly without thioredoxin as intermediate electron carrier showing a wider disulfide substrate specificity. These observations were used to develop a rapid and simple assay of the enzyme. The reaction had certain characteristics resulting from the use of a high concentration of the general sulfhydryl reagent DTNB as substrate. As shown in Fig. 1 the assay was only linear with time for a few minutes. This phenomenon is observed at different enzyme and substrate concentrations and can be explained most simply by a secondary chemical modification of thiol groups which inactivated the enzyme in parallel with an NADPH-dependent catalytic reaction at the active site. With purer fractions the deviation from linearity was less pronounced. In order to measure the enzyme activity we have only used the increase in absorbance during the 1st and 2nd min under strictly standardized conditions. The assay is then highly reproducible and proportional to the amount of enzyme in the range 2 to 15 milliunits.

The second assay of thioredoxin reductase (Method 2) utilized the reduction of calf liver thioredoxin by NADPH and thioredoxin reductase. The reduced form of thioredoxin is reoxdized by disulfide from insulin (Reaction 2) and the production of sulfhydryl groups in insulin was measured after the reaction had been stopped by addition of 6 M guanidine hydrochloride. This assay, which should be more specific for thioredoxin reductase, was used in parallel with the DTNB assay during early steps in the preparations to ensure that a thioredoxin reductase was purified. A standard curve for the insulin reduction assay is shown in Fig. 2. This assay was linear between 0.4 and 1.6 milliunits of enzyme and thus generally more sensitive than the DTNB assay. With more than 1.6 milliunits of thioredoxin reductase the free insulin B...
chain precipitated during the incubation and the final absorbance at 412 nm was higher than 2.0.

**Purification of Thioredoxin Reductase from Calf Liver**

In a previous paper the preparation of the reduced form of calf liver thioredoxin in high yield was described (11). The assay of calf liver thioredoxin requires thioredoxin reductase and the different fractions from the thioredoxin preparation could be used also for purification of thioredoxin reductase and a summary of the preparation method developed is given in Table I. The overall yield in the preparation is low because the methods are optimized to give good yields of thioredoxin (11). Furthermore, the DEAE-cellulose chromatography resulted in the separation of two peaks of thioredoxin reductase activity. A first peak of thioredoxin reductase which represented around 30% of the applied enzyme activity was eluted at a low salt concentration (0.03 M) and was not further studied. Evidence that this represents a different molecular form of the enzyme has subsequently been obtained. The second main peak of thioredoxin reductase was eluted at 0.15 M sodium acetate and overlapped the thioredoxin activity which showed a peak at 0.13 M salt. The main pool from the DEAE-cellulose chromatography (Fraction IV) thus contained both thioredoxin and thioredoxin reductase. The separation of thioredoxin and thioredoxin reductase was obtained by Sephadex G-50 chromatography. Two further steps using TEAE-cellulose and Sephadex G-200 chromatography could then be applied to further purify thioredoxin reductase. The enzyme after Sephadex G-200 (Fraction VII) was purified 1100-fold from the crude extract and was estimated to be of better than 30% purity by polyacrylamide gel electrophoresis (20). Further attempts to purify Fraction VII by hypatite or CM-cellulose chromatography were unsuccessful and resulted in only small increases in specific activity.

**Purification of Thioredoxin Reductase from Calf Thymus**

Calf thymus crude extracts contained thioredoxin reductase with approximately the same specific activity as crude extracts of liver. A preparation method designed to obtain a good yield of the enzyme was developed. Table II summarizes a typical purification of thioredoxin reductase from calf thymus. As with liver extracts two peaks of thioredoxin reductase were obtained by the DEAE-cellulose chromatography and only the major peak at 0.15 M sodium acetate was used for further purifications. Sephadex G-100 chromatography of Fraction V from calf thymus is shown in Fig. 3. The peak fraction in that chromatogram with a specific activity of 6.36 units/mg was used for characterization and represented a 2800-fold purification of the enzyme. This enzyme preparation was estimated to be of better than 30% purity by polyacrylamide gel electrophoresis (20).

**Properties of Purified Enzyme**

**Stability** – The most purified fractions of thioredoxin reductase could be stored at 0.2 to 1 mg/ml in 0.05 M Tris/Cl, pH 7.5, 1 mM EDTA at -20°C for months with less than 10% loss of activity with DTNB. Repeated freezing and thawing resulted in a considerable decrease in activity with calf liver thioredoxin and insulin without much effect of the activity with DTNB. After incubation with 2 mM dithiothreitol followed by extensive dialysis against 0.05 M Tris/Cl, pH 7.5, 1 mM EDTA the original activity with calf liver thioredoxin was restored suggesting that inactivation occurred through formation of disulfide-bonded enzyme aggregates which could be reversed by reduction.

**Activity with DTNB and NADPH** – The apparent \( K_m \) value of DTNB as substrate for thioredoxin reductase was determined as shown in Fig. 4 at a saturating concentration of NADPH. Maximal activity was obtained at 5 mM DTNB and an apparent \( K_m \) value of 1.5 mM was calculated. Reduction of 5 mM DTNB was absolutely dependent upon NADPH and with concentrations from 0.03 to 10 mM a constant enzyme activity was observed ruling out inhibition by excess NADPH. At 14 \( \mu \)M NADPH the initial velocity was 71% of that produced by 0.2 mM NADPH. Due to the rapid consumption of NADPH, accurate measurements at lower concentrations were not possible but the results showed that the apparent \( K_m \) value was below 10 \( \mu \)M. The stoichiometry of the reduction of DTNB was studied as shown in Fig. 5 at limiting concentrations of

![Fig. 4. The dependence of thioredoxin reductase activity on the concentration of DTNB. The standard conditions of the DTNB assay (Method 1) were used except that the final concentration of DTNB was varied as indicated. Fraction VII of calf liver thioredoxin reductase, 5.5 milliunits were used in all experiments.](image-url)

![Fig. 5. Stoichiometry of the reduction of 5 mM DTNB by NADPH in the presence of thioredoxin reductase. The amount of NADPH added was varied as indicated and the nanomoles of TNB produced was calculated from the absorbance at 412 nm. The DTNB assay conditions with 11 milliunits of Fraction VII from calf live thioredoxin reductase was applied.](image-url)
NADPH. The results supported the mechanism proposed in Reaction 3

\[
\text{NADPH} + H^+ + \text{DTNB} \rightarrow \text{NADP}^+ + 2 \text{TNB}
\]

in that 1 mol of NADPH reduced 1 mol of disulfide in DTNB to produce 2 mol of thionitrobenzoate in a reaction where all NADPH was consumed. The effect of sodium arsenite on the activity with 5 mM DTNB was studied as shown in Table III. Arsenite inhibited the activity of the enzyme and produced a 93% inhibition at 0.5 mM.

**Activity with Calf Liver Thioredoxin** — A natural electron acceptor for thioredoxin reductase is the disulfide bond of calf liver thioredoxin. The reduced form of calf liver thioredoxin previously isolated contained four sulfhydryl groups and aggregated upon oxidation *in vitro* (11). A preparation of calf liver thioredoxin at 6 mg/ml which had been stored at 20° and undergone repeated freezing and thawing cycles contained less than 0.1 µmol thiol/mmol of thioredoxin as determined with DTNB (19). The reduction of this material with NADPH in the presence of thioredoxin reductase showed an unusual biphasic behavior as shown in Fig. 6. A study of the stoichiometry of the reaction showed that 2 mol of NADPH are required to produce 4 mol of sulfhydryl groups in thioredoxin.

This result suggests that initially few molecules of active thioredoxin are able to reduce inactive disulfide-cross-linked thioredoxin molecules in a cascade effect which finally leads to reduction of all disulfides in the preparation. The oxidized calf liver preparation furthermore was shown to contain high molecular weight aggregates which largely chromatographed with the void volume of a Sephadex G-50 column. Treatment with 2 mM dithiothreitol for 30 min almost entirely converted the material into the native reduced thioredoxin (11) which eluted with a \( K_v \) value (21) of 0.25 on Sephadex G-50.

When the *in vitro* oxidized thioredoxin was used in the insulin reduction assay the activity was very low. Prolonged incubation times, however, resulted in more activity suggesting that activation of thioredoxin occurred in parallel with the reduction of insulin disulfides. After treatment of the oxidized thioredoxin with dithiothreitol a 7-fold activation was obtained with a 20-min assay, and the reduction of insulin was proportional to incubation time.

The apparent \( K_v \) value for activated calf liver thioredoxin was determined in the insulin reduction assay as shown in Fig. 7 and was calculated to be around 5 µM. Since the supply of calf liver thioredoxin was limited, the experiments with the insulin assay were routinely carried out at a concentration of 10 µM.

**Requirement for Protein Disulfide Reduction**

The reduction of protein disulfide bonds by NADPH was absolutely dependent upon the presence of thioredoxin as shown in Table IV. As little as 3.6 pmol of calf liver thioredoxin could be measured by this assay through recycling to produce 5.5 pmol of sulfhydryl groups in insulin, demonstrating the very efficient disulfide reduction in this system. Other disulfide-containing compounds were tested as electron donors with thioredoxin reductase in this protein disulfide reduction system. Thioredoxin from *E. coli* gave the same activity as calf liver thioredoxin provided that a 10-fold higher concentration was used. Very low activity was obtained with phage 14 thioredoxin. No net reduction of insulin disulfides could be obtained with either oxidized glutathione or lipoic acid. The

**TABLE III**

<table>
<thead>
<tr>
<th>Arsenite</th>
<th>Activity</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>100</td>
</tr>
<tr>
<td>2 x 10⁻³</td>
<td>0.104</td>
<td>46</td>
</tr>
<tr>
<td>1 x 10⁻³</td>
<td>0.040</td>
<td>18</td>
</tr>
<tr>
<td>5 x 10⁻³</td>
<td>0.016</td>
<td>7</td>
</tr>
<tr>
<td>5 x 10⁻²</td>
<td>0.006</td>
<td>3</td>
</tr>
</tbody>
</table>

**FIG. 6. Oxidation of NADPH by *in vitro* oxidized calf liver thioredoxin, *Escherichia coli* and T4 thioredoxin in the presence of thymus thioredoxin reductase. Two cuvettes contained in a final volume of 0.50 ml: 0.1 M potassium phosphate buffer, pH 7.0, 2 mM EDTA, and 3 x 10⁻⁴ M NADPH. To one cuvette, which was placed in the reference position in the spectrophotometer was added either 0.05 nmol of calf liver thioredoxin, •—•, or 8 nmol of *E. coli* thioredoxin, ○—○; or 8.7 nmol of phage T4 thioredoxin, ×—×. The reaction was started by addition of thymus thioredoxin reductase (11 milliunits) to both cuvettes and the increase in absorbance at 340 nm was recorded against time.

The sulphydryl groups produced at the highest concentration of glutathione (1.9 x 10⁻⁴ M) were consistent with stoichiometric reduction of glutathione without any net reduction of disulfides of insulin.

**Cross-reactivity with Thioredoxin from Other Species**

Thioredoxin-S₈ from *E. coli* was rapidly reduced by NADPH in the presence of calf thioredoxin reductase (Fig. 6), with the same kinetic behavior as in the presence of *E. coli* thioredoxin reductase. Thioredoxin from *E. coli* has a single disulfide bridge in its oxidized form and the kinetics of reduction was thus entirely different from the corresponding experiment with the completely *in vitro* oxidized calf liver thioredoxin described above. The phage 14 coded thioredoxin, which is induced in *E. coli* after phage T4 infection (22, 23) is rapidly reduced by *E. coli* thioredoxin reductase and NADPH. As
Fig. 7. Dependence of insulin disulfide reduction of NADPH and thioredoxin reductase on the concentration of calf liver thioredoxin. In this experiment, Method 2 was used with varying final concentration of dithiothreitol-activated calf liver thioredoxin and 13 milliunits of thymus thioredoxin reductase. The result is expressed as nanomoles of thiol formed from insulin disulfides during incubation for 20 min at 37°C.

Table IV

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Final concentration</th>
<th>Disulfide reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf liver thioredoxin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calf liver thioredoxin</td>
<td>3 x 10^-3</td>
<td>5.5</td>
</tr>
<tr>
<td>Calf liver thioredoxin</td>
<td>7.5 x 10^-3</td>
<td>14.7</td>
</tr>
<tr>
<td>Calf liver thioredoxin</td>
<td>1.6 x 10^-3</td>
<td>29.6</td>
</tr>
<tr>
<td>Escherichia coli thioredoxin</td>
<td>8.8 x 10^-4</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>E. coli thioredoxin</strong></td>
<td><strong>9.6 x 10^-4</strong></td>
<td><strong>19.0</strong></td>
</tr>
<tr>
<td><strong>E. coli thioredoxin</strong></td>
<td><strong>1.0 x 10^-3</strong></td>
<td><strong>125.0</strong></td>
</tr>
<tr>
<td><strong>T4 thioredoxin</strong></td>
<td><strong>7.5 x 10^-3</strong></td>
<td><strong>0</strong></td>
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<tr>
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<td><strong>8.4 x 10^-3</strong></td>
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<td>GSSG</td>
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</tr>
<tr>
<td>GSSG</td>
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<td>39</td>
</tr>
<tr>
<td>Oxidized lipoic acid</td>
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</tr>
<tr>
<td>Oxidized lipoic acid</td>
<td>1.7 x 10^-5</td>
<td>0</td>
</tr>
<tr>
<td>Oxidized lipoic acid</td>
<td>1.9 x 10^-5</td>
<td>7.1</td>
</tr>
</tbody>
</table>

shown in Fig. 6, T4 thioredoxin-S, was only very slowly reduced by calf thioredoxin reductase at a concentration where E. coli thioredoxin is rapidly reduced.

Yeast thioredoxin II which is homologous to E. coli thioredoxin (6) was also an excellent substrate for calf thioredoxin reductase (Fig. 8). Addition of insulin to the completely reduced yeast thioredoxin resulted in further consumption of NADPH consistent with a rapid reduction of insulin disulfides.

No NADPH-oxidation activity with calf thioredoxin reductase was obtained with 1 mM L-cystine or by 1 x 10^-5 M of the disulfide-containing peptide thioredoxin C-(1-37) from E. coli thioredoxin (24).

Relation of Enzyme Activity with DTNB and Calf Liver Thioredoxin

The two assay methods for calf thioredoxin reductase used in this study are both based on the NADPH-dependent reduction of disulfide bonds. Method 1 uses DTNB, an artificial but readily available substrate, whereas Method 2 used the natural substrate which is difficult to prepare. The rates of disulfide reduction with these two substrates have been compared quantitatively. In one experiment 1.2 milliunits of thymus thioredoxin reductase (0.2 μg) and 1 x 10^-8 M calf liver thioredoxin produced 62 nmol of sulfhydryl groups in insulin during a 20-min incubation at 37°C. The corresponding reduction of DTNB at 25°C in the standard assay calculated per 20 min would be 25 nmol of sulfhydryl groups. Thus, the two methods gave similar turnover numbers for the enzyme. The higher activity with thioredoxin may be explained by the difference in temperature and reagents in the two assays. At 25°C both methods give essentially the same molar activities (data not shown).

Another important aspect of the use of DTNB assay to measure calf thioredoxin reductase concerns its specificity when applied to early steps in purification. Since protein and low molecular weight sulfhydryl groups react with DTNB, two chromatography steps before glutathione was removed from extracts by ammonium sulfate precipitation of the DTNB assay showed 30 to 40% higher values than the specific calf liver thioredoxin-coupled assay. After the first column chromatography step the two assay methods showed an essentially constant ratio. As noted above oxidation of sulfhydryl groups on thioredoxin reductase had little influence on the activity with DTNB.

Discussion

In the present study thioredoxin reductase was purified from crude extracts of liver and thymus by using the initial rate of the NADPH-dependent reduction of DTNB as an assay. This assay has the great advantage of being rapid and simple. Furthermore, it allows measurements of thioredoxin reduc-
a labile enzyme that is complicated to assay. Since the unspecific thiol reagent DTNB was used as substrate it was essential to demonstrate the specificity of the DTNB assay for thioredoxin reductase. This was accomplished by comparing the relative specific activities with DTNB and the natural substrate calf liver thioredoxin during the different purification steps. In crude extracts, glutathione and glutathione reductase apparently contribute to the NADPH-dependent reduction of DTNB. When the bulk of glutathione was removed from extracts by dialysis or ammonium sulfate precipitation the DTNB assay appeared specific for thioredoxin reductase.

The calf liver and thymus thioredoxin reductase showed a wider substrate specificity for the disulfide substrate than the corresponding E. coli enzyme (1). Thus, the thioredoxin from E. coli and yeast were rapidly reduced. This may be of practical importance in assays of thioredoxin reductase with a coupled thioredoxin-dependent reaction since E. coli thioredoxin is easier to purify and has no additional sulfhydryl groups that may form mixed disulfides. Thioredoxin reductase from calf liver may also be useful to assay thioredoxin from plant or algal cells provided these contain a thioredoxin that is cross-reacting. The finding that DTNB at 5 mM concentration functions as a substrate for the bovine thioredoxin reductase with approximately the same initial rate of reduction, as when 10 μM calf liver thioredoxin was used, is in accordance with the wider substrate specificity. However, L-cystine or insulin were not reduced. Oxidized glutathione was slowly reduced but at this stage of purification it is difficult to distinguish between true cross-reactivity and some contamination with traces of glutathione reductase.

The thioredoxin system from bovine cells resembles the well characterized thioredoxin system from E. coli (26) in many respects. The thioredoxin from E. coli and calf are both small heat-stable proteins and have a homologous amino acid sequence around the active center disulfide bridge suggesting that they have evolved from a common ancestral protein. An important difference is that thioredoxin from calf (11) and rat Novikoff tumor cells (27) both contain additional sulfhydryl groups that upon in vitro O2 oxidation form intermolecular mixed disulfides leading to aggregation and inactivation. The aggregated and completely oxidized calf thioredoxin was a poor substrate for thioredoxin reductase. The reduction with NADPH showed an interesting non-first order kinetics that strongly suggested a process of autocatalysis, i.e. inactive aggregated thioredoxin molecules were activated after reduction with thioredoxin-(SH)2. If the formation of this aggregated form of thioredoxin occurs in vivo it may represent an important regulatory function in the total disulfide-reducing capacity of the thioredoxin system within a cell. Other possible functions of these extra thiol groups of mammalian thioredoxins may be to link thioredoxin via disulfide bonds to other cellular structures. Alternatively, they may readily form mixed disulfides with GSH and thereby possibly be involved in control of activity.

The reduction of protein disulfides and L-cystine by the mammalian thioredoxin system requires the presence of thioredoxin as intermediate electron carrier. A highly efficient nucleotide-dependent reduction of protein disulfides by NADPH was demonstrated in this study with as little as 3 × 10-6 M thioredoxin. Since calf liver contains 50 to 100 mg (5 to 10 μmol) of thioredoxin/kg of tissue, wet weight (11), the overall intracellular concentration should be in the range of 5 to 10 μM; at this level, the thioredoxin system may play an important role in oxidation of disulfide bonds in proteins and enzymes. The results of this study show efficient reduction of insulin disulfides by the thioredoxin system. In previous studies rapid reduction by thioredoxin of exposed disulfides in human fibrinogen and fibrin (28) as well as the glycoprotein hormone human chorionic gonadotropin and its two subunits, α and β, (29) has been demonstrated.

Previously an NADPH-dependent enzyme called protein disulfide reductase (12) (EC 1.6.4.4) was identified in pea seeds. Studies by Tietze on disulfide reduction in rat liver (13, 14) and the results of this paper strongly suggest that the protein disulfide reductase is identical with the thioredoxin system. Tietze used DEAE-cellulose chromatography (13, 14) to separate two enzyme activities from a dialyzed postmicrosomal supernatant of rat liver that promoted disulfide reduction with NADPH but not with NADH. These activities were a glutathione reductase and an NADPH-dependent disulfide reductase of broad specificity. In addition rat liver contained a GSH-disulfide oxidoreductase (transhydrogenase) which catalyzed the reduction of both protein and non-protein disulfide substrates by GSH in the presence of NADPH and an exogenous source of glutathione reductase. The NADPH-dependent disulfide reductase activity which reduced insulin, DTNB and L-cystine was further separated in two fractions by chromatography on Sephadex G-150: a high molecular weight heat-labile fraction, that reduced DTNB but not insulin and was sensitive to inhibition by arsenite, and a low molecular weight fraction which was stable to heating at 70° for 30 min and which, when added to the heat-labile fraction, restored the capacity to reduce insulin disulfides. The low molecular weight fraction was inactive with NADPH and any disulfide substrate. Tietze (15) noted the similarity of this enzyme system and the thioredoxin system of E. coli. The results of this paper provide firm evidence that the protein disulfide-reducing capacity studied by Tietze is identical with the thioredoxin system of mammalian liver. We have recently purified thioredoxin and thioredoxin reductase from normal rat liver, and shown that this system has the same properties as the calf enzyme system.

The activity of a protein disulfide reductase that by several criteria is identical with the thioredoxin system described in this paper, has been undertaken by Appfel and Walker (15) in different murine tumor cells, exudates, and fluids. The protein disulfide reductase assayed with DTNB showed a striking increase from 5 to 20 times in tumor cells whereas a smaller increase was observed for glutathione reductase and no difference from normal cells was observed for the GSH-protein disulfide oxidoreductase. Furthermore, the protein disulfide reductase appeared in plasma of tumor-bearing mice in contrast to normal sera which had no activity. Studies of blocking of reactive —SH groups by disulfide reagents and inactivation of the protein disulfide reductase strongly suggested that also proliferation and immunogenicity of these murine tumors depended on reactive sulfhydryl groups and the activity of protein disulfide reduction (15).

Thioredoxin will not form any stable complex with thioredoxin reductase when the two purified proteins are mixed in solution and the two proteins separate completely when a crude extract of liver is subjected to gel filtration. Furthermore, both proteins may be inactivated by oxidation of sulfhydryl groups. Measurements of the NADPH-dependent disulfide reducing activity of a fraction should therefore be performed after incubation with dithiothreitol followed by di-
Disulfide Reduction of Bovine Thioredoxin Reductase

We have recently developed a radioimmunassay for calf liver thioredoxin which enables highly sensitive measurements of this protein in subcellular fractions. The results of these studies show that a fraction of the cellular thioredoxin is associated with membrane structures or enclosed in vesicles, from which it can be released by detergents. The finding of two peaks of thioredoxin reductase activity by DEAE-cellulose chromatography may be consistent with different molecular forms of the enzyme and is now being further investigated. These studies and further purifications of the thioredoxin reductase described in this paper have been greatly simplified by our recent development of an efficient affinity chromatography method with the use of N6-(6-aminohexyl)-adenosine-2',5'-bisphosphate-Sepharose.²

Acknowledgments—Mr. Nils-Erik Engström participated in part of these investigations. The excellent technical assistance of Mrs. Gunilla Olovson and Mrs. Maja-Lena Granqvist is gratefully acknowledged.

REFERENCES

5. I. Brutt, A. Holmgren, and M. Luthman, to be submitted for publication.

SUPPLEMENTAL MATERIAL

Table 1

Table 2

Note: All data are expressed as mean ± SEM. The significance of differences between groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. The data were analyzed using a statistical software package. P values less than 0.05 were considered statistically significant.

³ I. Brutt, A. Holmgren, and M. Luthman, to be submitted for publication.

Bovine thioredoxin system. Purification of thioredoxin reductase from calf liver and thymus and studies of its function in disulfide reduction.

A Holmgren


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