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Articles

Rat Liver Thioredoxin and Thioredoxin Reductase: Purification and Characterization†

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ABSTRACT: A reproducible scheme has been developed for the preparation of rat liver thioredoxin and thioredoxin reductase (EC 1.6.4.5) by using assays based on reduction of insulin and 5,5′-dithiobis(2-nitrobenzoic acid), respectively. Both proteins were purified to homogeneity, as judged from polyacrylamide gel electrophoresis. Thioredoxin had a molecular weight of 12000 and contained about 110 amino acids including 4 half-cystines and an NH_2-terminal valine. Peptide maps of reduced and carboxymethylated thioredoxin showed that the protein had the active center sequence -Cys-Gly-Pro-Cys-Lys-Met- characteristic of thioredoxins also from procaryotes. Prolonged air oxidation of fully reduced thioredoxin created inactive, aggregated disulfide-containing molecules. Thioredoxin reductase showed a subunit molecular weight of 58000 and a native molecular weight of 116000. The enzyme was highly specific for NADPH with a K_m of 6 μM. It contained FAD as prosthetic group and was sensitive to inhibition by arsenite. Thioredoxin reductase had a K_m of 2.5 μM for rat and calf liver thioredoxin and a K_{cat} of 3000 min^{-1}.

The thioredoxin system, thioredoxin, thioredoxin reductase, and NADPH, is a widespread thiol-dependent electron transport system [reviews by Williams (1976) and Holmgren (1980, 1981)]. Thioredoxin was originally purified from *Escherichia coli* as a dithiol hydrogen donor for the enzyme ribonucleotide reductase (Thelander & Reichard, 1979) and for enzymes catalyzing the reduction of sulfoxides and sulfate [see Holmgren (1980, 1981)]. In addition, the thioredoxin system promotes the reduction of certain protein disulfides by NADPH through a combination of reactions 1 and 2.

$$\text{thioredoxin-}S_2 + \text{NADPH} + H^+ \xrightarrow{\text{thioredoxin reductase}} \text{thioredoxin-}(SH)_2 + \text{NADP} \quad (1)$$

$$\text{thioredoxin-}(SH)_2 + \text{protein-}S_2 \rightarrow \text{thioredoxin-}S_2 + \text{protein-}(SH)_2 \quad (2)$$

E. coli thioredoxin-S_2 (M_r 11700) has been extensively characterized (Homgren, 1981). It contains an oxidation-reduction active cystine disulfide (-Cys_{32}-Gly-Pro-Cys_{35}-) in a unique protrusion of the three-dimensional structure (Holmgren et al., 1975). Thioredoxin reductase (EC 1.6.4.5) from *E. coli* (M_r 70000) is a dimer of two apparently identical subunits, each containing FAD and an oxidation-reduction active disulfide (Williams, 1976).

Mammalian thioredoxins and thioredoxin reductase have so far remained largely uncharacterized. The lack of readily available standardized assay systems and the inactivation and apparent aggregation of the proteins in the absence of thiols have complicated studies (Holmgren, 1980). Homogeneous preparations of thioredoxin have been obtained from the *Novikoff* ascites rat tumor (Herrmann & Moore, 1973), by using a partially purified tumor ribonucleotide reductase, and from calf liver (Engström et al., 1974), by an assay utilizing the reduction of insulin disulfide by NADPH in the presence of a partially purified calf thioredoxin reductase. It is not known if *Novikoff* tumor thioredoxin is identical with the rat liver thioredoxin, which has beforehand only been partially purified (Larson & Larsson, 1972).

Thioredoxin reductase has been purified to about 95% homogeneity from the Novikoff ascites rat tumor (Chen et al., 1977) and also partly purified from rat liver (Larsson, 1973) and calf liver and thymus (Holmgren, 1977). The tumor enzyme was reported to have an isoelectric point of 5.1 by isoelectric focusing (Chen et al., 1978). Variant forms with isoelectric points of 4.9 and 4.7 were observed in adult rat liver extracts, but the implications of these findings remain unclear

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since the rat liver enzyme was not obtained pure.

In this paper we describe a reproducible preparation method for rat liver thioredoxin and thioredoxin reductase that yields homogeneous proteins. We have developed standardized and simple assay methods for thioredoxin and thioredoxin reductase that should be applicable to all mammalian thioredoxin systems. A physicochemical and kinetic characterization of the enzymes is reported.

Experimental Procedures

Materials. Pooled frozen livers (−20 °C) from adult rats (Sprague-Dawley albino) were gifts from Drs. K. Hall, H. Eriksson, and A. Löfström, Karolinska Institutet, Stockholm. Thioredoxin reductase from calf thymus was purified as described (Holmgren, 1977; Holmgren & Lyckeborg, 1980). Thioredoxin from *E. coli* B was prepared by method 2 (Holmgren & Reichard, 1967). Thioredoxin from calf liver was isolated as described by Engström et al. (1974).

Sephadex G-25, G-50, and G-150 and 2′5′-ADP–Sepharose 4B were from Pharmacia AB (Sweden). (ω-Aminohexyl)-agarose was from Miles (Israel), and DEAE-cellulose and CM-cellulose were from Whatman (England). Bovine insulin (26 units/mg) was a product of vitrum (Sweden). Crystallized bovine serum albumin was from British Drug House Ltd. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin (TPCK–trypsin) and chymotrypsin were purchased from Worthington Biochemical Corp. Molecular weight calibration proteins for $NaDodSO_4$[1] gel electrophoresis were Combithek from Boehringer Mannheim, West Germany. DTNB[1] and DTT[1] were from Calbiochem (Switzerland). NADPH, NADH, FAD, FMN, GSSG,[1] alloxan, and menadione were from Sigma Products. Diaflo UM2 membranes came from Amicon Corp. (Holland). Spectrapor membranes (no. 3) for dialysis were from Spectrum Medical Industries, Ltd. Dansyl[1] chloride and dansyl amino acids were from Mann, and polyamide thin-layer plates were obtained from Cheng Chin Trading Co., Taiwan, Formosa. Iodoacetic acid was from Sigma and iodo[^{14}C]acetic acid from the Radiochemical Centre, Amersham, England. Other reagents were of analytical grade and obtained from commercial sources.

Protein Determination. The A_{230}/A_{260} method of Kalb & Bernlohr (1977) was used. Absorbance was measured in a Zeiss PMQIII spectrophotometer.

Concentration of Protein Solutions. Dilute pools of thioredoxin and thioredoxin reductase in the first column steps were concentrated by ultrafiltration with N_2 at 4 °C in Diaflo cells (Amicon) with UM2 membranes. In the later steps, thioredoxin fractions could be lyophilized without loss of activity. Thioredoxin reductase fractions were concentrated by vacuum filtration with ultrafilters (Membranengesellschaft, Göttingen, West Germany).

Determination of Free SH Groups. This was done spectrophotometrically by using 1 mM DTNB in 6 M guanidine hydrochloride and 0.2 M Tris-HCl, pH 8.0, using a molar extinction coefficient of 13 600 at 412 nm (Ellman, 1959).

Thioredoxin Reductase. Two methods were used to determine thioredoxin reductase activity. Method 1: DTNB assay. Rat liver thioredoxin reductase catalyzed the NADPH-dependent reduction of the disulfide bond in DTNB (Holmgren, 1977). The assay mixture contained 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 0.2 mM NADPH, 0.2 mg/mL bovine serum albumin, 1% ethanol, and 5 mM DTNB. Enzyme (1–50 μL) was added to 500 μL of mixture at 25 °C in a semimicrocuvette, and the increase in absorbance at 412 nm between the first and second minute was determined with a recording Zeiss PMQIII spectrophotometer. Activity was calculated as micromoles of NADPH oxidized per minute according to $\Delta A_{412} \times 0.5/(13.6 \times 2)$ since 1 mol of NADPH yields 2 mol of thionitrobenzoate (Holmgren, 1977).

Method 2: Spectrophotometric insulin reduction. The assay mixture contained 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 80 μM insulin (0.5 mg/mL), 0.2 mM NADPH, and 3 μM *E. coli* thioredoxin-S_2. Enzyme was added to a cuvette containing 0.50 mL of mixture at 25 °C. The reaction rate was followed from the oxidation of NADPH at 340 nm; activity was calculated as micromoles of NADPH oxidized per minute from the relation $\Delta A_{340} \times 0.5/6.2$.

Thioredoxin Assays. Two methods were used. Method 3: Spectrophotometric insulin reduction. The same assay mixture (0.5 mL) as in method 2 was used in the presence of 10 μg of rat liver thioredoxin reductase or calf thymus thioredoxin reductase at 25 °C. Thioredoxin (1–20 μL) giving a final concentration of 0.5–2 μM was added, and the reaction was followed at 340 nm. The assay was standardized by using *E. coli* thioredoxin-S_2 (2 μM).

Method 4: Micromethod of insulin reduction. The incubation mixture contained, in a final volume of 120 μL, 0.25 mg of insulin, 0.6 μmol of EDTA, 10 μmol of *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid buffer, pH 7.6, 0.11 μmol of NADPH, and 0.5 μg of rat liver thioredoxin reductase. Thioredoxin (5–20 μL of 20 μg/mL) was added, and tubes were incubated at 37 °C for 20 min. The reaction was broken by addition of 0.50 mL of 6 M guanidine hydrochloride–50 mM Tris-HCl (pH 8.0)–10 mM DTNB. The absorbance at 412 nm was measured, and the activity was calculated from $A_{412} \times 0.62/(13.6 \times 2)$ as micromoles of NADPH oxidized since 1 mol of NADPH corresponds to 2 mol of sulfhydryl groups (reaction 2).

Glutathione Reductase Assay. The assay conditions were 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 0.2 mM NADPH, and 1 mM GSSG. The assay volume was 0.5 mL, and the reaction was followed at 340 nm. Activity was expressed as micromoles of NADPH oxidized per minute.

Polyacrylamide Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis (Ornstein, 1964; Davis, 1964), using the pH 8.4 buffer system was carried out in 10% polyacrylamide gels. The gels were stained with 0.25% Coomassie Brilliant Blue and destained with 7% acetic acid–10% methanol. Sodium dodecyl sulfate gel electrophoresis was carried out by a slab-gel technique as described by O'Farrel (1975).

Amino Acid Analysis. Lyophilized samples of rat liver thioredoxin or thioredoxin reductase (1–3 nmol) were hydrolyzed with 0.5 mL of 6 M HCl containing 0.5% phenol for 24 h at 110 °C in vacuo. The amino acid composition was determined with a Beckman Model 121-M amino acid analyzer. Half-cystine was determined as cysteic acid after performic acid oxidation by the method of Hirs (1967). Tryptophan was determined spectrophotometrically (Bencze & Schmid, 1957).

NH_2-Terminal Analysis. The NH_2-terminal amino acid of thioredoxin was determined by the dansyl method of Gray & Hartley (1967). Dansyl amino acids were identified by two-dimensional thin-layer chromatography on polyamide sheets in four solvent systems (Woods & Wang, 1967; Crowshaw et al., 1967).

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; Cys(Cm), carboxymethylcysteine; GSSG, oxidized glutathione; Tris, tris(hydroxymethyl)aminomethane.

Table I: Purification of Thioredoxin and Thioredoxin Reductase from 200 g of Rat Liver

fraction	protein (mg)	thioredoxin protein (mg)	thioredoxin act. (units)[a]	thioredoxin sp act. (units/mg)	thioredoxin reductase protein (mg)	thioredoxin reductase act. (units)[b]	thioredoxin reductase sp act. (units/mg)
crude extract	38 000		3800	0.002		305	0.008
pH 5 supernatant	18 000		3300	0.004		220	0.012
dialyzed ammonium sulfate precipitate	6 000		2400	0.010		200	0.033
DEAE-cellulose		350	2000	0.14	760	125	0.165
Sephadex G-50		10.3	1500	3.60			
CM-cellulose		4.9	900	4.60			
2′5′-ADP–Sepharose					10.5	105	10.0
(ω-aminohexyl)agarose					1.6	55	35.0

[a] Micromoles of NADPH oxidized per minute (method 4, insulin reduction, excess thioredoxin reductase). [b] Micromoles of NADPH oxidized per minute (method 1, DTNB reduction).

Carboxymethylation of Thioredoxin and Peptide Maps. Lyophilized rat liver thioredoxin, 2.0 mg, was dissolved in 400 μL of 6 M guanidine hydrochloride, 0.2 M Tris-HCl, and 2 mM EDTA, pH 8.0, and reduced by addition of 10 μL of 0.5 M DTT. After incubation under N_2 for 1 h at 37 °C, 150 μL of 0.1 M iodo[^{14}C]acetic acid (2400 cpm/nmol) was added. After incubation for 1 h at 37 °C, the carboxymethylated thioredoxin was isolated by chromatography on a column of Sephadex G-25 (10 mL) equilibrated with 60 mM NH_4HCO_3 and lyophilized.

Carboxymethylated thioredoxin (4 nmol) was digested with trypsin or chymotrypsin (1:75 w/w in 60 mM NH_4HCO_3 for 2 h at 37 °C) and lyophilized. Peptides were separated by high-performance liquid chromatography on a column of μBondapak C_{18} (Waters) by using a gradient of 2% methanol–0.4% NH_4HCO_3 (solvent A) and 80% methanol–0.36% NH_4HCO_3 (solvent B) with 10–80% B in 80 min. The flow rate was 1.1 mL/min by using a LDC chromatograph. Peptide maps on thin-layer plates were prepared as described (Holmgren, 1979).

Results

Purification of Thioredoxin and Thioredoxin Reductase. The first steps in the purification of thioredoxin and thioredoxin reductase were identical, except for minor modifications, to the method previously used for calf liver (Engström et al., 1974). Compounds that interfered in the assay systems, mainly glutathione, were removed by these first, common steps.

Rat liver (200 g) was homogenized and acid and heat treated and the supernatant fraction precipitated to 85% saturation with solid ammonium sulfate. The precipitate formed was dialyzed and chromatographed on DEAE-cellulose. Thioredoxin reductase activity tested by the DTNB assay eluted as a main peak at 0.13 M NaCl. A second small (<10%) peak of DTNB activity of variable intensity in different preparations was present in the flow-through fractions (0–0.03 M NaCl). This peak that coincided with glutathione reductase activity was pooled and purified separately (see below). It gave highly purified glutathione reductase.

Thioredoxin activity was located at 0.15 M NaCl. Depending on the relative separation of thioredoxin and thioredoxin reductase, either they were pooled together or a separate reductase pool was made. This was then taken directly to the 2′5′-ADP–Sepharose chromatography.

Sephadex G-50 and CM-cellulose Chromatographies of Thioredoxin. Attempts to purify the oxidized form of thioredoxin were not successful. Thioredoxin easily aggregated and appeared as multiple peaks in the chromatograms after air oxidation. This was effectively overcome by incubation with DTT under nitrogen prior to chromatography steps.

The concentrated pool from the DEAE-cellulose chromatography, incubated with 2 mM DTT for 30 min at 25 °C, was applied to a column of Sephadex G-50 (6 × 120 cm) equilibrated with 60 mM NH_4HCO_3. Thioredoxin and thioredoxin reductase were well separated; the thioredoxin reductase appeared with the main protein peak in the void volume. Fractions containing thioredoxin were pooled and lyophilized. The lyophilized pool was dissolved in 5 mL of H_2O, pH adjusted to 7 with 0.1 M NH_4OH, and incubated with 2 mM DTT for 30 min at 25 °C under N_2 and 0.5 mL of 0.1 M sodium acetate, pH 5.16, was added. It was then applied to a column of CM-cellulose (1 × 10 cm) equilibrated with 10 mM sodium acetate, pH 5.16. The column was eluted with a linear gradient (60 mL of each) of 10 and 100 mM sodium acetate, pH 5.16. Thioredoxin-containing fractions, appearing around 60 mM sodium acetate, were combined, adjusted to pH 7.5 by 1 M NH_4OH, and lyophilized. Thioredoxin was normally homogeneous after this step. If not, a rechromatography on Sephadex G-50 (column size 1 × 120 cm) in 60 mM NH_4HCO_3 served to remove traces of impurities.

A summary of the preparation of rat liver thioredoxin is given in Table I. An apparent 2000-fold purification with a yield of 24%, based on activity measurements of the crude extract, was achieved.

2′,5′-ADP–Sepharose and (ω-Aminohexyl)agarose Chromatographies of Thioredoxin Reductase. Thioredoxin reductase fractions from Sephadex G-50 or from DEAE-cellulose were dialyzed against 50 mM Tris-HCl–1 mM EDTA buffer, pH 7.5, and applied to a column (1.5 × 10 cm) of 2′5′-ADP–Sepharose 4B (Pigiet & Conley, 1977). After the column was washed with the same buffer, thioredoxin reductase was eluted with a 5-mL pulse of 10 mM $NADP^+$ or, preferrably, by 0.20 M potassium phosphate (pH 7.5)–1 mM EDTA.

Glutathione reductase fractions from the DEAE-cellulose column were purified separately on 2′5′-ADP–Sepharose. This enzyme was eluted with 10 mM $NADP^+$, but not with 0.20 M potassium phosphate.

Thioredoxin reductase concentrated by ultrafiltration and dialyzed against 50 mM Tris-HCl–1 mM EDTA, pH 7.5, was applied to a column of (ω-aminohexyl)agarose (1 × 12 cm) and eluted with a linear gradient of sodium chloride, 0–0.8 M (100 mL of each), in 50 mM Tris-HCl–1 mM EDTA, pH 7.5. Thioredoxin reductase appeared at 0.2 M NaCl. The enzyme was concentrated and dialyzed against 50 mM Tris-HCl–1 mM EDTA, pH 7.5, and stored frozen at −20 °C.

Thioredoxin reductase was normally electrophoretically pure after the (ω-aminohexyl)agarose step. If not, an additional 2′5′-ADP–Sepharose step using enzyme applied in 10 mM

Table II: Amino Acid Composition of Rat Liver Thioredoxin and Thioredoxin Reductase

amino acid	assumed integral value for thioredoxin	assumed integral value for thioredoxin reductase[a]
lysine	13	52
histidine	1	12
arginine	0	23
aspartic acid	11	49
threonine	4	35
serine	5	24
glutamic acid	15	55
proline	3	23
glycine	7	54
alanine	12	38
half-cystine[b]	4	14
valine	11	43
methionine	2	7
isoleucine	3	32
leucine	7	48
tyrosine	2	18
phenylalanine	9	21
tryptophan	1[c]	ND[d]
total	110	

[a] Based on a subunit molecular weight of 58 000. [b] Determined as cysteic acid after performic acid oxidation (Hirs, 1967). [c] Determined spectrophotometrically (Bencze & Schmid, 1957). [d] ND, not determined.

potassium phosphate–1 mM EDTA, pH 7.0, and eluted with 0.20 M potassium phosphate–1 mM EDTA, pH 7.4, served to remove traces of contaminating proteins.

The purification of thioredoxin reductase is summarized in Table I. An apparent 4000-fold purification with a yield of 18% was achieved.

Properties of Rat Liver Thioredoxin. (*A*) *Purity.* Analytical nondenaturing gel electrophoresis of thioredoxin after incubation with DTT showed the presence of a single protein band.

(*B*) *NH_2 Terminal.* Valine was shown to be the only NH_2-terminal amino acid.

(*C*) *Molecular Weight.* The K_{av} value of rat liver thioredoxin-$(SH)_2$ was 0.25 on a calibrated column of Sephadex G-50 in 50 mM Tris-HCl–1 mM EDTA, pH 7.5. This was identical with the value for *E. coli* thioredoxin-S_2, indicating a molecular weight of 12 000 (Holmgren, 1968). The molecular weight determined by $NaDodSO_4$ gel electrophoresis (15% gel) in the presence or absence of DTT was 12 000 with *E. coli* thioredoxin and cytochrome *c* as markers.

(*D*) *Amino Acid Composition.* The amino acid composition of rat liver thioredoxin is given in Table II. The molecule contained all common amino acids except arginine, including four half-cystine residues.

(*E*) *Stability of the Reduced Form of Thioredoxin in Vitro.* Thioredoxin was reduced by excess DTT and isolated free from DTT on a column of Sephadex G-25 equilibrated with 60 mM NH_4HCO_3. The SH content was followed in a sample of thioredoxin (173 μM) during incubation at 25 °C in a parafilm covered tube. The disappearance of SH groups was a rather slow process, with two of the initially four half-cystines remaining after 35 h.

(*F*) *Properties of in Vitro Oxidized Rat Liver Thioredoxin.* The activity of a sample of oxidized thioredoxin containing no free SH groups had decreased to less than 1% as a substrate for thioredoxin reductase (methods 3 and 4). The oxidized thioredoxin was analyzed by chromatography on Sephadex G-50; all material was eluted in the void volume of the column, indicating the formation of aggregates. On native polyacrylamide gel electrophoresis of the oxidized material, a number of bands appeared whereas $NaDodSO_4$ gel electrophoresis in the absence of DTT showed only material at 12 000, strongly indicating the formation of intramolecular disulfides. Incubation of the oxidized thioredoxin with 2 mM DTT for 10 min restored about 80% of the activity and the native molecular weight as determined by chromatography on Sephadex G-50.

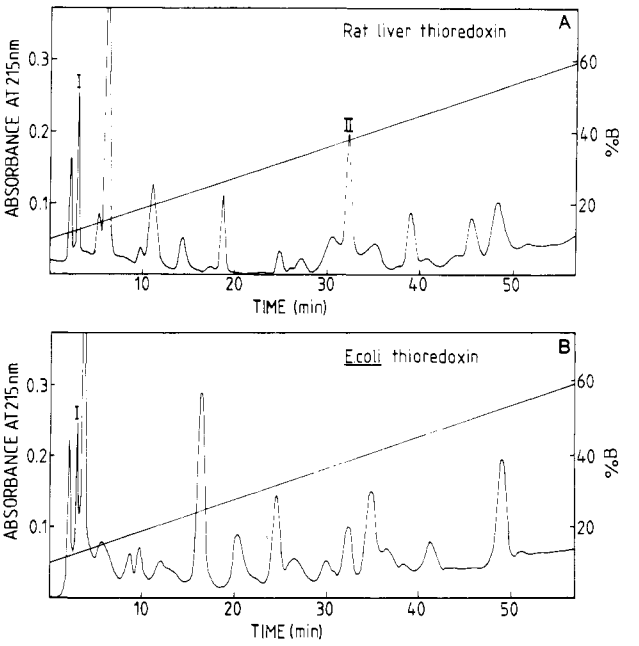

FIGURE 1: Separation of a chymotryptic digest of 4 nmol of [^{14}C]-carboxymethylated rat liver thioredoxin (A) and *E. coli* thioredoxin (B) by high-performance liquid chromatography. A column of μBondapak C_{18} (Waters) was used with 2% methanol and 0.4% NH_4HCO_3 (solvent B). A gradient from 10 to 80% B was run in 80 min with a flow rate of 1.1 mL/min. The peptide labeled I in both chromatograms was radioactive and is derived from the active center.

(*G*) *Ultraviolet Spectrum.* The spectrum of rat liver thioredoxin at pH 7 in the ultraviolet region showed no other chromophores than tryptophan and tyrosine. Incubations of thioredoxin with 1 mM DTT in 60 mM NH_4HCO_3 in air resulted in a change in the ultraviolet spectrum of the protein after desalting. The absorbance at 260 and 310 mm were increased relative to 280 nm, probably due to oxidation of tryptophan residues by oxygen and DTT [see Baccanari (1978)]. This was avoided by the addition of EDTA in the buffer or by incubation with DTT under N_2.

(*H*) *Active Center.* Peptide maps of chymotryptic digests of [^{14}C]carboxymethylated rat liver thioredoxin and *E. coli* thioredoxin were prepared by high-performance liquid chromatography (Figure 1). Two radioactive peaks corresponding to [^{14}C]carboxymethylcysteine appeared in the chromatogram. The active center peptide from rat liver thioredoxin was identified from its identical position in the chromatogram of *E. coli* thioredoxin. The peptide had the following amino acid composition: Cys (Cm) 2.1, Pro 0.8, Gly 1.0, Met 0.7, and Lys 1.0. We conclude that the active center of rat liver thioredoxin is identical with that of *E. coli* thioredoxin (Holmgren, 1980): Cys-Gly-Pro-Cys-Lys-Met-. This was also confirmed by autoradiography of peptide maps of tryptic digests of rat liver and *E. coli* thioredoxin prepared on thin-layer plates (Gleason & Holmgren, 1981).

Properties of Rat Liver Thioredoxin Reductase. (*A*) *Purity.* Analytical polyacrylamide gel electrophoresis of the final material showed the presence of a single protein band, which after elution contained thioredoxin reductase activity.

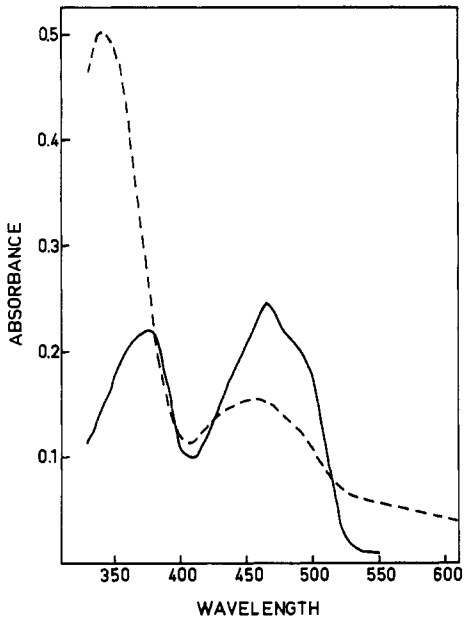

FIGURE 2: Visible absorption spectrum of rat liver thioredoxin reductase. (—) Thioredoxin reductase (10 μM) in 0.1 M potassium phosphate, pH 7.0, and 1 mM EDTA; (---) thioredoxin reductase reduced with NADPH (0.10 mM).

(*B*) *Molecular Weight.* $NaDodSO_4$ polyacrylamide gel electrophoresis of thioredoxin reductase showed one protein band, the position of which corresponded to a subunit molecular weight of 58 000. In the absence of DTT a similar subunit molecular weight was found, demonstrating that the subunits were not linked by disulfide bonds. On a column of Sephadex G-150, equilibrated with 0.05 M Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1 M NaCl, thioredoxin reductase was eluted between bovine serum albumin (M_r 68 000) and glucose-6-phosphate dehydrogenase from yeast (M_r 110 000), close to glutathione reductase (M_r 100 000). Thioredoxin reductase thus consists of two identical or similar subunits with a native molecular weight of 116 000.

(*C*) *Flavin Content.* The purified thioredoxin reductase had a visible absorption spectrum typical for flavoproteins, similar to the spectrum of *E. coli* thioredoxin reductase It had two intensity maxima, at 380 and 465 nm. The ratio between A_{280} and A_{460} was 8.0 (cf. *E. coli* thioredoxin reductase 4.8) (Thelander, 1967). Reduction of the enzyme by 10 equiv of NADPH resulted in bleaching of the visible part of the spectrum (Figure 2). Precipitation of thioredoxin reductase with acid ammonium sulfate greatly decreased its activity. The inactivated apoenzyme was partly reactivated by addition of FAD but not by FMN. We therefore conclude that the prosthetic group in rat liver thioredoxin reductase is FAD.

(*D*) *Amino Acid Composition.* The amino acid composition of rat liver thioredoxin reductase is given in Table II.

(*E*) *Activity with DTNB and NADPH.* The apparent K_m of NADPH at a saturating concentration of DTNB (5 mM) was determined to be 6 μM. NADH even in mM concentrations gave no activity. The apparent K_m for DTNB was determined to be 0.66 mM in the presence of 100 μM NADPH.

(*F*) *Activity with Thioredoxin.* The activity of rat liver thioredoxin reductase with thioredoxin from various species is shown in Table III. Bovine and rat liver thioredoxins were reduced with the same apparent K_m values, whereas the K_m of *E. coli* T-S_2 was 14-fold higher. The K_{cat} however, was similar for all three thioredoxins examined and even somewhat higher for DTNB.

Table III: Kinetic Constants for Rat Liver Thioredoxin Reductase[a]

substrate	K_m value (μM)	K_{cat} (min^{-1})
rat liver thioredoxin	2.5	3000
calf liver thioredoxin	2.5	3000
E. coli thioredoxin	35	3000
DTNB[b]	660	4000

[a] The experiments were made in 0.50 mL of 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.2 mM NADPH, and 80 μM insulin at 25 °C. Varying concentrations of thioredoxin were used with 5.5 nM thioredoxin reductase. Reaction rates were calculated from the ΔA_{340nm} (method 2). [b] Determined by method 1.

Table IV: Substrate Specificity of Rat Liver Thioredoxin Reductase[a]

substrate	act. (nmol of NADPH min^{-1} mL^{-1}) at concn (mM) of 0.1	0.4	1.0	10.0
alloxan	1.6		8.3	
menadione	0.4		0.6	
GSSG			0	0
oxidized lipoamide			0.6	1.4
insulin	0	0		
L-cystine			0.4	0.6
DTNB[b]	4.9		11.6	

[a] Indicated concentrations of various substrates were added to two cuvettes containing 0.1 M potassium phosphate, 2 mM EDTA, pH 7.0, and 0.2 mM NADPH in 0.50 mL. The first cuvette served as the blank, and to the other was added thioredoxin reductase to a final concentration of 4 nM. Activity was determined from the decrease in absorbance at 340 nm. [b] Determined from the increase in absorbance at 412 nm.

(*G*) *Inhibition of Thioredoxin Reductase by DTNB in the Presence of Thioredoxin.* The thioredoxin reductase dependent reduction of DTNB by NADPH measured spectrophotometrically at A_{412} nm showed an unexpected inhibition by addition of thioredoxin. Thus, in assays with 1.5 nM rat liver thioredoxin reductase and 120 μM DTNB, the addition of 3.8 μM rat liver or calf liver thioredoxin resulted in more than 90% inhibition within 2 min. With 5 mM DTNB, the inhibition was a considerably slower process. *E. coli* thioredoxin at 6 μM showed no detectable effect.

(*H*) *Activity with Other Substrates.* The reactivity of thioredoxin with some disulfide and nondisulfide substrates was examined as shown in Table IV. Alloxan was a good nondisulfide substrate for thioredoxin reductase as previously found for the calf thymus enzyme (Holmgren & Lyckeborg, 1980).

(*I*) *Sensitivity to Arsenite.* Sodium arsenite decreased the activity of thioredoxin reductase in the DTNB assay to 70% at 0.01 mM and to 4% at 0.1 mM.

Discussion

This paper describes the isolation of homogeneous rat liver thioredoxin and thioredoxin reductase in good yields. The purification of thioredoxin was based on its ability to reduce the disulfide bonds of insulin by NADPH in the presence of thioredoxin reductase. Thioredoxin reductase was measured by using its capacity to reduce DTNB by NADPH. These methods allow the isolation of the thioredoxin system independent of other enzymes such as ribonucleotide reductase that is not readily available. The purification of thioredoxin reductase by affinity chromatography on 2′5′-ADP–Sepharose

proved to be highly effective, particularly the elution of the enzyme free from glutathione reductase by 0.2 M potassium phosphate. This may possibly reflect the high specificity of thioredoxin reductase toward NADPH in contrast to glutathione reductase that is also active with NADH (Williams, 1976).

Thioredoxins from rat liver and *E. coli* have the same molecular weights. Both proteins show similar activity as disulfide reductases with insulin as substrate (Holmgren, 1980). The amino acid sequence of the active center disulfide

$$\text{—S—S—}$$

is -Cys-Gly-Pro-Cys- and has been shown to be identical in *E. coli* (Holmgren, 1968), yeast (Hall et al., 1971), *Anabaena sp.* (Gleason & Holmgren, 1981), *Corynebacterium nephridii* (Meng & Hogenkamp, 1981), and rat liver thioredoxins.

In contrast to *E. coli* and yeast thioredoxins, mammalian thioredoxins contain additional half-cystine residues apart from the active center disulfide (Herrmann & Moore, 1973; Engström *et al.*, 1974). These structural SH groups make the purification and handling of all mammalian thioredoxins difficult in comparison with *E. coli* thioredoxin. Air oxidation of reduced rat liver thioredoxin results in the formation of inactive molecules, which have intramolecular disulfides. The oxidation state of the structural half-cystine residues in vivo is so far unknown.

Thioredoxin reductases from *E. coli* and yeast have native molecular weights of around 70 000 (Williams, 1976). Both enzymes are dimers and have one molecule of FAD per subunit. Thioredoxin reductase from rat liver is also a dimer containing two FAD but has a molecular weight of 116 000. Apparently, this considerably larger size of the rat liver enzyme is typical of mammalian thioredoxin reductases (Chen et al., 1977; Holmgren & Lyckeborg, 1980).

The rat liver thioredoxin reductase showed a broader substrate specificity in comparison with the highly specific *E. coli* enzyme. Thus, the rat liver enzyme reduced *E. coli* thioredoxin and also calf liver thioredoxin. The wider substrate specificity is also shown by the high reactivity with DTNB. Combinations of rat liver thioredoxin and DTNB as substrates resulted in inactivation of rat liver thioredoxin reductase. Although the mechanism behind this effect is unknown, it suggests that DTNB chemically modifies the thioredoxin reductase when it is binding a mammalian thioredoxin containing structural half-cystines.

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