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Structure and Enzymatic Functions of Thioredoxin Refolded by Complementation of Two Tryptic Peptide Fragments†

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ABSTRACT: The physicochemical and catalytic properties of thioredoxin-T′ are described. This complemented protein structure consists of a 1:1 complex between the inactive fragments thioredoxin-T-(1–73) and thioredoxin-T-(74–108). These are generated by selective trypsin cleavage at Arg-73 in lysine-modified and denatured *Escherichia coli* thioredoxin. Thioredoxin-T′ was a slowly formed but stable complex with an apparent K_D below 10^{-8} M. The tryptophan fluorescence spectrum and the CD spectrum were very similar to those of native thioredoxin; some conformational differences were detected by gel chromatography and radioimmunoassay. Thioredoxin-T′-S_2 was a substrate for NADPH and thioredoxin reductase and had 1–2% of the activity of native thioredoxin. This low relative activity was the result of a major increase in the K_m value. Thioredoxin-$(SH)_2$ was a hydrogen donor for *E. coli* ribonucleotide reductase with about 3% relative activity. These results for thioredoxin-T′ are correlated with the known three-dimensional structure of thioredoxin. The microenvironment around Arg-73 that is close to the active disulfide appears to be of critical importance for the interactions of thioredoxin with thioredoxin reductase and ribonucleotide reductase.

Thioredoxin-S_2[1] contains an intramolecular cystine disulfide bridge (–Cys_{32}-Gly-Pro-Cys_{35}–) as the catalytically important group (Holmgren, 1968). This disulfide is reduced to a dithiol with NADPH in a reaction catalyzed by the specific flavoprotein thioredoxin reductase [for a review, see Holmgren (1980)]. Thioredoxin-$(SH)_2$ is an efficient disulfide reductase and is reoxidized in reactions such as those shown in Figure 1. The assays for thioredoxin activity are thus based on the cyclic oxidoreduction of thioredoxin in the presence of NADPH and thioredoxin reductase and a disulfide acceptor. A novel and so far largely unknown function of *Escherichia coli* thioredoxin is to be the host-coded essential subunit of phage T7 DNA polymerase (Mark & Richardson, 1976), required for virus DNA replication in vivo and in vitro.

Previous studies of the structure and function of thioredoxin have been aimed at an understanding of its molecular mechanism of action. Thus, the complete primary structure of the 108 amino acid residues of thioredoxin-S_2 from *E. coli* has been determined (Holmgren, 1968). The three-dimensional structure of thioredoxin-S_2 has been solved to 2.8-Å resolution by X-ray crystallographic techniques (Holmgren et al., 1975). In addition, we previously identified two systems of peptide fragments that can be used to reconstitute thioredoxin-S_2 by noncovalent complementation (Holmgren, 1972a; Slabý & Holmgren, 1975). Cleavage of thioredoxin with cyanogen bromide at the single methionine residue (Met-37) yields the two peptide fragments thioredoxin-C-(1–37) and thioredoxin-C-(38–108) that upon mixing form thioredoxin-C′. Selective cleavage by trypsin of thioredoxin, where all the lysines have been blocked by citraconic anhydride, splits the molecule at the single arginine residue (Arg-73). The resulting peptide fragments, after removal of citraconyl groups by mild acid hydrolysis, thioredoxin-T-(1–73) and thioredoxin-T-(74–108), interact specifically to give thioredoxin-T′ (Slabý & Holmgren, 1975).

Thioredoxin-T′ and thioredoxin-C′ both give full immunoprecipitation activity with rabbit antibodies against native thioredoxin and have a low but significant enzymatic activity with thioredoxin reductase (Slabý & Holmgren, 1975). The component peptides of these complexes are enzymatically inactive. However, thioredoxin-T-(74–108) and also thioredoxin-C-(38–108) were strong inhibitors of the precipitation reaction of native thioredoxin-S_2 with its antibodies. This demonstrated that one of the major antigenic determinants of thioredoxin is contained in the thioredoxin-T-(74–108) amino acid sequence. Furthermore, the results show that this COOH-terminal fragment has the capacity of nucleated folding into a structure similar to that which it normally occupies in native thioredoxin. This conclusion has been strongly supported by the three-dimensional structure of thioredoxin-S_2

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[1] Abbreviations used: thioredoxin-S_2 (T-S_2) and thioredoxin-$(SH)_2$ [T-$(SH)_2$], the oxidized and reduced forms of thioredoxin, respectively; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid). Peptide fragments have been designated by an adoption of the rules of the IUPAC–IUB Commission on Biochemical Nomenclature. Fragments obtained after CNBr cleavage are denoted by C and after selective cleavage with trypsin are denoted by T. The reconstituted noncovalent complexes are denoted thioredoxin-C′ and thioredoxin-T′ (Slabý & Holmgren, 1975).

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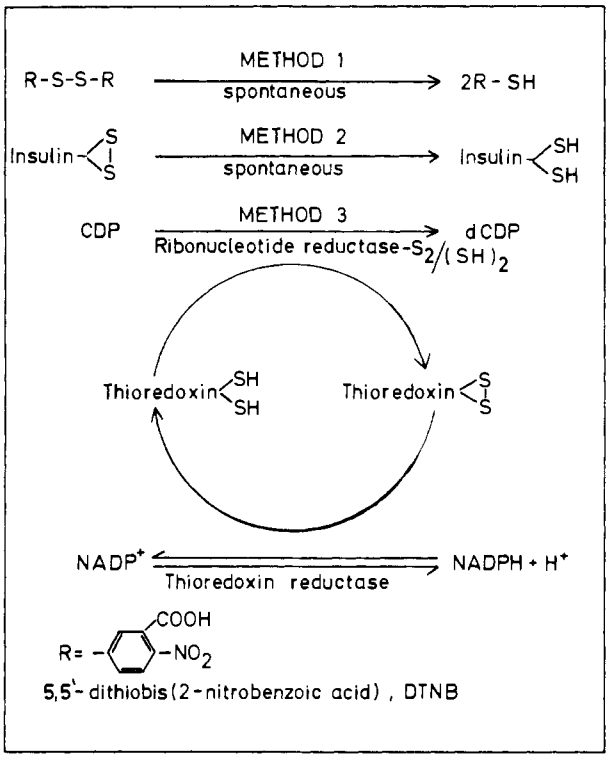

FIGURE 1: Cyclic oxidoreduction reactions that measure the catalytic activity of thioredoxin. Method 1 is used to follow spectrophotometrically at 412 nm the thioredoxin-dependent reduction of DTNB. Method 2 is used to follow the thioredoxin-dependent cleavage of insulin disulfides. Method 3 is used to measure the conversion of [^{3}H]CDP to [^{3}H]dCDP catalyzed by *E. coli* ribonucleotide reductase; this enzyme contains an oxidation–reduction active disulfide participating in the catalytic mechanism (Thelander, 1974).

(Holmgren et al., 1975), in which the COOH-terminal third of the molecule forms an independent folding unit, consisting of two strands of antiparallel β-pleated sheet and a terminal α helix.

In this paper the physicochemical and enzymatic properties of thioredoxin-T′ are described. The results show that thioredoxin-T′ is a strong complex with a folding similar to native thioredoxin both in its oxidized and in its reduced form. The possible folding mechanism and the catalytic activity of thioredoxin-T′ are related to the three-dimensional structure of thioredoxin-S_2. In an accompanying paper (Holmgren & Slabý, 1979), the results of studies of thioredoxin-C′ and its component peptides are reported.

Experimental Procedure

Material. Thioredoxin was prepared from *E. coli* B by method 2 as described previously (Holmgren & Reichard, 1967) and had a disulfide content of 73 nmol/A_{280} unit as measured by oxidation of NADPH in the presence of thioredoxin reductase, indicating homogeneity. Thioredoxin reductase from *E. coli* after Sephadex G-100 chromatography (Thelander, 1967) and ribonucleotide reductase (Brown et al., 1969) were enzyme preparations available in this laboratory. NADPH and DTNB were from Sigma Chemical Co. Bovine insulin, 25.3 units/mg, was purchased from Vitrum, Stockholm, Sweden. Urea was an ultrapure preparation from Schwartz/Mann; acrylamide and *N,N′*-methylenebis(acrylamide) were from Eastman Kodak Co. Sephadex G-50 and Sephadex G-25 were from Pharmacia; dithiothreitol was from Calbiochem; bovine serum albumin and citraconic anhydride were from British Drug House. DEAE-cellulose chromatography was performed on Whatman DE-32. Sodium [^{125}I]-iodide for protein iodination was a carrier-free preparation from the Radiochemical Centre, Amersham, England. Sheep antirabbit γ-globulin (sheep 64/73) was obtained from Statens Bakteriologiska Laboratorium, Stockholm, Sweden. Rabbit antithioredoxin antibodies and the γ-globulin fractions were obtained as described previously (Holmgren & Sjöberg, 1972).

Preparation of Peptide Fragments. Thioredoxin-T-(1–73) and thioredoxin-T-(74–108) were prepared by citraconylation of thioredoxin, followed by trypsin digestion, which was stopped by addition of soybean trypsin inhibitor.[2] The two peptides were separated on a column of Sephadex G-50 in 50% acetic acid as described earlier (Slabý & Holmgren, 1975). This treatment also removes the citraconyl groups to regenerate all amino groups. The deblocked peptide fragments were further purified by chromatography on Sephadex G-25 in 0.5% NH_4HCO_3. The purity of the fragments and their concentration were determined by amino acid analysis (Slabý & Holmgren, 1975).

Preparation of Thioredoxin-T′. Thioredoxin-T′ was prepared by overnight incubation at 25 °C of 1 μmol of thioredoxin-T-(1–73) and thioredoxin-T-(74–108) in 2 mL of 0.5% NH_4HCO_3. The mixture was then applied to a column of Sephadex G-50 in 0.5% NH_4HCO_3 (2 × 130 cm) at 4 °C, and thioredoxin-T′ was isolated.

Enzymatic Assays for Thioredoxin Activity (See Figure 1). Method 1 (DTNB Assay). Thioredoxin was assayed with thioredoxin reductase by using DTNB as the electron acceptor (Slabý & Holmgren, 1975). Each of the two cuvettes contained in a final volume of 500 μL the following: 50 μL of 1.0 M Tris-HCl, pH 8.0; 10 μL of 0.2 M EDTA; 20 μL of 0.01 M DTNB in 96% ethanol; 10 μL of the bovine serum albumin, 5.0 mg/mL. To one cuvette was added thioredoxin or peptide fragments, and the other cuvette served as a blank. The reaction was started by addition of thioredoxin reductase to both cuvettes, and the reduction of DTNB was followed at 412 nm.

Method 2 (Insulin Assay). Thioredoxin or thioredoxin-T′ was first reduced by NADPH and thioredoxin reductase and then reoxidized by insulin (Holmgren et al., 1978). Each of two cuvettes contained 500 μL of 10 mM Tris-HCl–1 mM EDTA, pH 6.4 or 8.7, and 10 μL of 10 mM NADPH. Thioredoxin was added to the blank cuvette. After a zero-time reading at 340 nm, thioredoxin reductase (0.1 μM) was added to both cuvettes, and the increase in absorbance was followed until no further oxidation of NADPH was observed. Insulin, 40 μL of 1.6 mM, was then added to both cuvettes. The rate of the insulin reduction was followed from the change in absorbance at 340 nm.

Method 3 (Ribonucleotide Reductase Assay). The activity of thioredoxin as hydrogen donor for ribonucleotide reductase was determined from the conversion of [^{3}H]CDP to [^{3}H]dCDP in the presence of excess NADPH and thioredoxin reductase as described in detail elsewhere (Holmgren et al., 1978).

Protein Determination. The protein concentration was determined from the absorbance at 280 and 310 nm with a molar absorptivity of 13 700 M^{-1} cm^{-1} for native thioredoxin, thioredoxin-T′, and thioredoxin-T-(1–73) (Holmgren & Reichard, 1967). The concentration of thioredoxin-T-(74–108)

[2] Addition of bovine pancreatic trypsin inhibitor, Trasylol, Bayer Co., to stop the trypsin digestion resulted in the generation of inhibitory thioredoxin peptide fragments. This is due to the presence of sodium merthiolate, which is added as a preservative to the solutions of Trasylol. Sodium merthiolate is a highly effective inhibitor at less than 1×10^{-7} M for thioredoxin reductase from *E. coli* or calf thymus. It is a competitive inhibitor of thioredoxin reductase when thioredoxin is the substrate.

was determined from amino acid analysis.

Amino Acid Analysis. Peptides were hydrolyzed for 24 h in 6 M HCl at 110 °C in vacuo. The content of amino acids was determined on a Beckman 120B amino acid analyzer as described previously (Slabý & Holmgren, 1975).

Spectrophotometric Determinations. A Zeiss PMQ3 spectrophotometer equipped with an automatic sample exchanger and a Servogor 541 recorder was used. Cells with a 1-cm light path taking 500 μL were used routinely.

Gel Electrophoresis. Polyacrylamide disc gel electrophoresis was carried out with a Shandon SAE 2734 apparatus by the method of Ornstein (1964) and Davis (1964). A discontinuous buffer system with pH 8.9 was used. The electrophoresis was run in 10% polyacrylamide gels at 30 V/cm for 40 min. The gels were then stained overnight by 0.25% Coomassie brilliant blue in 12% trichloroacetic acid and destained with 12% trichloroacetic acid.

Radioimmunoassay. A competition radioimmunoassay for thioredoxin using ^{125}I-labeled thioredoxin and a double-antibody technique was used (Holmgren et al., 1978). The incubation mixture contained in a final volume of 330 μL the following: 100 μL of rabbit antithioredoxin γ-globulin diluted 1:1000, 30 μL of [^{125}I]thioredoxin tracer (~5 ng), and 100 μL of various dilutions of thioredoxin, thioredoxin-T′, or the peptide fragments. After incubation for 4 h at 37 °C, 100 μL of sheep antiserum to rabbit γ-globulin (diluted 1:5) was added, and the tubes were left for 16 h at 4 °C. After centrifugation, the free [^{125}I]thioredoxin in 100 μL of the supernatant was determined with a Packard liquid scintillation counter.

Fluorescence Measurements. Fluorescence spectra were determined in 0.5 mL of 0.10 M phosphate–1 mM EDTA, pH 7.0, at 25 °C with 1-cm cells. The emission spectrum of thioredoxin-S_2, thioredoxin-T′-S_2 or thioredoxin-T-(1–73)-S_2 was recorded first, the protein was then reduced by addition of 3 μL of 0.5 M dithiothreitol, and the spectrum was redetermined after 3 min. The fluorescence emission spectra were recorded with a Zeiss Spectrofluorometer ZFM 4, with double monochromators equipped with a wavelength motor and a Honeywell Model Y 153 × 18 IV 23 recorder. Fluorescence was excited at 280 nm, and the emitted light was recorded from 290 to 450 nm. Solvent blanks were subtracted from the spectra, which are given uncorrected for monochromator and detector responses.

Circular Dichroism. CD spectra were measured with a Jasco Model J-20 spectropolarimeter. The cell path was 1 mm, and the protein concentrations were between 1.8 and 2.8 × 10^{-5} M. The mean residue weight of thioredoxin is 115. The ellipticity was computed as usual (Björk & Tanford, 1971).

Results

Preparation of Thioredoxin-T′. Deblocked thioredoxin-T-(1–73) and thioredoxin-T-(74–108) were separately rechromatographed on a column of Sephadex G-50 to give single symmetrical peaks. Thioredoxin-T′ was prepared by incubation of equimolar amounts of thioredoxin-T-(1–73) and thioredoxin-T-(74–108). The mixture was applied to a column of Sephadex G-50 (Figure 2A), and a main peak of thioredoxin-T′ was obtained, in 50–70% yield. Varying amounts of aggregates and nonfunctional peptide material were removed in the chromatography. Rechromatography of thioredoxin-T′ yielded a single symmetrical peak.

Thioredoxin-T′ could also be isolated by chromatography on DEAE-cellulose using a linear gradient of 0.02–0.50 M potassium phosphate, pH 7.0. The peak was eluted at 0.15 M salt. Thus, once formed, thioredoxin-T′ showed no tendency to dissociate in the chromatographic procedures, showing that it is a stable complex.

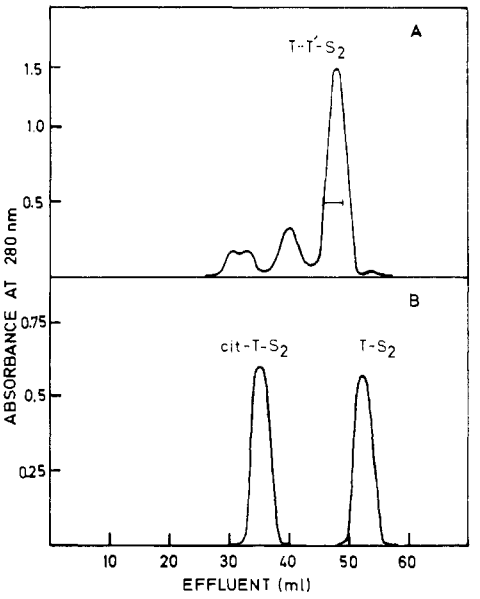

FIGURE 2: (A) Sephadex G-50 chromatography of thioredoxin-T′. Thioredoxin-T-(1–73) and thioredoxin-T-(74–108) were incubated in a 1:1 ratio (0.5 μmol of each) overnight and applied to a Sephadex G-50 column (0.9 × 135 cm) equilibrated with 0.5% NH_4CHO_3, pH 8.0, at 4 °C. Thioredoxin-T′ (T-T′) appears as the main peak in the chromatogram. (B) Sephadex G-50 chromatography of thioredoxin and citraconylated thioredoxin. The chromatography conditions were the same as those described in (A). The first peak corresponds to citraconylated thioredoxin (cit-T-S_2) and the second corresponds to native thioredoxin (T-S_2).

Table I: Gel Chromatography of Thioredoxin-T′ and the Peptide Fragments[a]

sample	K_{AV} value[b]
thioredoxin-S_2	0.34
citraconylated thioredoxin-S_2	0.10
thioredoxin-T′-S_2	0.29
thioredoxin-T′-$(SH)_2$	0.22
thioredoxin-T-(1–73)-S_2	0.18
thioredoxin-T-(74–108)	0.37

[a] A column of Sephadex G-50 (0.9 × 140 cm) equilibrated with 0.5% ammonium bicarbonate was used. [b] K_{AV} values (Laurent & Killander, 1964) were calculated according to the equation $K_{AV} = (V_e - V_0)/(V_T - V_0)$ where V_e is elution volume of the protein and V_T is the total volume of the column. V_0 for this Sephadex G-50 column was determined to be 33% of V_T.

Characterization of Thioredoxin-T′ and the Fragments by Gel Chromatography. Gel chromatography on a calibrated column of Sephadex G-50 was used to measure elution volumes and to calculate K_{AV} values (Laurent & Killander, 1964) for thioredoxin-S_2, citraconylated thioredoxin, thioredoxin-T′, and the peptide fragments (Table I). Citraconylated thioredoxin, a totally inactive highly acidic molecule (Slabý & Holmgren, 1975), had the lowest K_{AV} value, indicating a random-coil structure with a Stokes radius larger than that of native thioredoxin-S_2 (Figure 2B). Thioredoxin-S_2 is known from the three-dimensional structure (Holmgren et al., 1975) to be an essentially spherical molecule with overall dimensions of 25 × 34 × 35 Å. Thioredoxin-T-(1–73) had a K_{AV} value which indicated that it is mainly a random coil. The somewhat high value for thioredoxin-T-(74–108) may reflect a lower molecular weight and a compact size consistent with some folding. The citraconylated peptide fragments had essentially the same K_{AV} values on Sephadex G-50 chromatography as the deblocked fragments. The formation of thioredoxin-T′-S_2

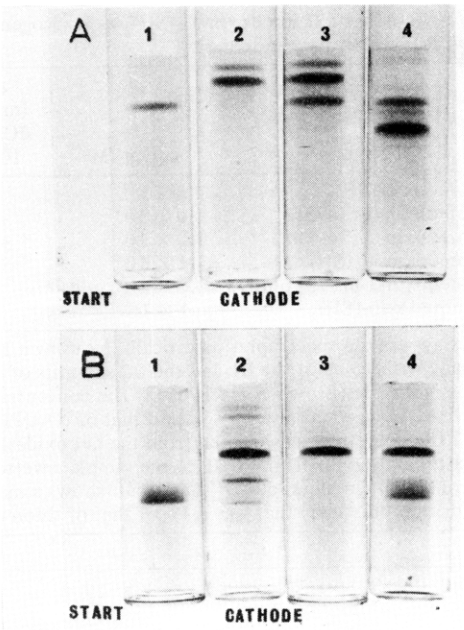

FIGURE 3: Polyacrylamide gel electrophoresis of thioredoxin-T′. (A) 10 μg of each of (1) native thioredoxin, (2) thioredoxin-T′, (3) mixed thioredoxin and thioredoxin-T′, and (4) mixed thioredoxin and thioredoxin-T-(1–73) was run on the polyacrylamide gels at pH 8.9. (B) 10 μg each of (1) native thioredoxin, (2) thioredoxin-T-(1–73), (3) thioredoxin-T′, and (4) mixed native thioredoxin and thioredoxin-T′ was run on gels containing 7 M urea at pH 8.9. The small peptide thioredoxin-T-(74–108) was not stained.

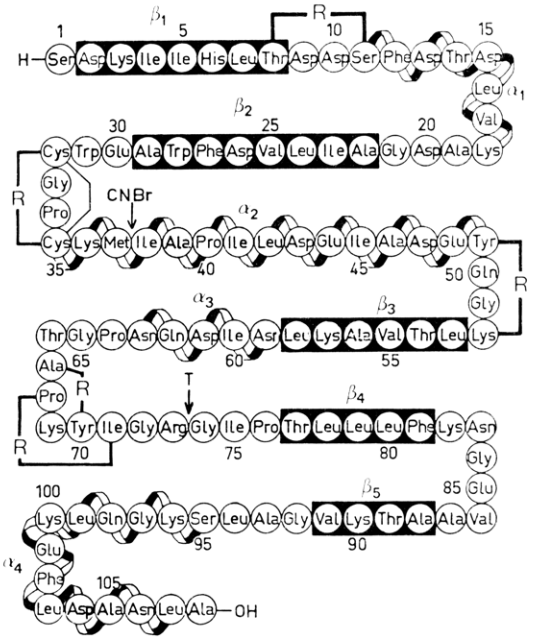

FIGURE 4: The amino acid sequence of thioredoxin-S_2 from *E. coli* including the secondary structure elements, α helices, β-pleated sheets, and reverse turns (R) as determined from the three-dimensional structure (Holmgren et al., 1975). The specific cleavage point at Arg-73 by trypsin (T) is indicated by an arrow. The cleavage at Met-37 by CNBr is also indicated by an arrow.

was accompanied by a folding to a thioredoxin-like structure as seen from the K_{AV} values of Table I. However, in all experiments the K_{AV} value of thioredoxin-T′-S_2 was seen to be significantly lower than that of thioredoxin-S_2. This is consistent with a more flexible structure of thioredoxin-T′, resulting in a larger effective Stokes radius.

Characterization by Polyacrylamide Gel Electrophoresis. This was performed in order to compare the behavior of the peptide fragments, thioredoxin-T′, and thioredoxin under native and denaturing conditions (Figure 3). Peptide-T-(74–108) could not be visualized in the gels since it was not stained despite the use of trichloroacetic acid in the staining procedure. Under nondenaturing conditions (pH 8.9), thioredoxin-S_2 showed a high anodical mobility consistent with its acidic isoelectric point of 4.5 (Holmgren, 1968). Thioredoxin-T-(1–73), which has a lower molecular weight and is even more acidic than thioredoxin (Figure 4), showed a lower mobility. This indicates that the fragment has a larger effective Stokes radius than native thioredoxin, corresponding to a random-coil structure. Furthermore, the fragment had the same mobility at pH 8.9 in 7 M urea, strongly suggesting that it was a random coil under native conditions and not a dimer. In contrast, thioredoxin had a markedly lower mobility in 7 M urea at pH 8.9, indicating the unfolding of the molecule. Indeed, as seen in Figure 3B, thioredoxin in 7 M urea showed a trailing forward, suggesting a finite equilibrium between a folded and unfolded form of the protein. Thioredoxin-S_2 is known to be quite resistant to unfolding in urea (Holmgren, 1972b). Thioredoxin-T′ moved faster than thioredoxin at pH 8.9 in native gels (Figure 3A). This behavior is expected since at pH 8.9 the COOH group of Arg-73 carries a negative charge not present in native thioredoxin. The preparations of thioredoxin-T′ contained also varying amounts of a second band with even faster mobility. This probably arises from partial deamidation of Asn-83 under the acidic conditions of preparation of the peptide fragments (Holmgren, 1968; Holmgren & Slabý, 1979). The gels show no trace of thioredoxin in the thioredoxin-T′ preparations. Furthermore, the band of thioredoxin-T′ was sharp, and no band corresponding to thioredoxin-T-(1–73) was seen in the thioredoxin-T′ preparation. This is consistent with a strong complex of thioredoxin-T′ without any detectable dissociation. In contrast, thioredoxin-T′ in 7 M urea showed only one band corresponding to thioredoxin-T-(1–73). The results demonstrate that thioredoxin-T′ is a noncovalent complex and that it is not stable under the denaturing conditions of 7 M urea.

Table II: Enzymatic Activity of Thioredoxin-T′ and Thioredoxin with Thioredoxin Reductase Measured by Reduction of DTNB

	enzymatic act.[a]	
thioredoxin-T′ (M)	$\Delta A_{412} \times \text{min}^{-1}$	% of thioredoxin
1×10^{-6}	0.015	1.07
4×10^{-6}	0.060	1.07
8×10^{-6}	0.165	1.47
2×10^{-5}	0.320	1.14
4×10^{-5}	0.560	1.00

[a] Activity was determined by method 1 (see Experimental Procedure). The concentration of thioredoxin reductase was 1.0×10^{-7} M. Thioredoxin showed an activity of $\Delta A_{412} \times \text{min}^{-1}$ of 0.140 at 1.0×10^{-7} M and the $\Delta A_{412} \times \text{min}^{-1}$ was proportional to thioredoxin concentration in the range from 0 to 1.0 $\Delta A_{412} \times \text{min}^{-1}$. A control sample of citraconylated thioredoxin that was subjected to the same 50% acetic acid chromatography as the peptide fragments showed 52% of the activity of native thioredoxin.

Enzymatic Activity. The two peptide fragments thioredoxin-T-(1–73) and thioredoxin-T-(74–108) of thioredoxin-T′ were completely inactive as substrates for thioredoxin reductase (1×10^{-7} M) at 1×10^{-5} M concentration (Figure 5). The purified complex thioredoxin-T′ had activity with thioredoxin reductase as measured by the reduction of DTNB with excess thioredoxin reductase (Table II). Thioredoxin-T′ showed around 1% of the activity of native thioredoxin in the range from 1×10^{-6} to 40×10^{-6} M. There

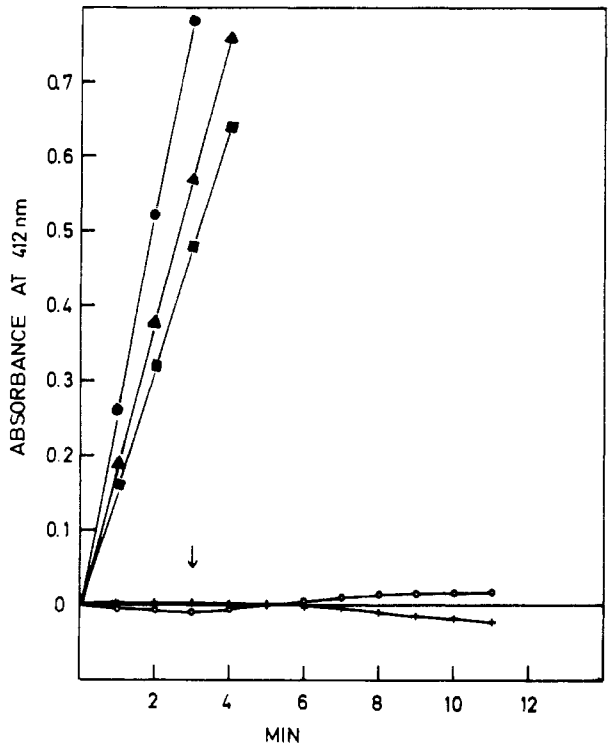

FIGURE 5: Enzymatic activity of thioredoxin-T′ and the peptide fragments with thioredoxin reductase. The DTNB assay (method 1) with 2×10^{-7} M thioredoxin reductase was used. Thioredoxin, 1×10^{-7} M (●); 1:1 mixture of thioredoxin-T-(1–73) and thioredoxin-T-(74–108), 1.2×10^{-4} M, preincubated 10 h at 25 °C and assayed at 1.1×10^{-5} M (▲); mixture of thioredoxin-T-(1–73), 1.1×10^{-5} M, and thioredoxin-T-(74–108), 1.6×10^{-5} M, preincubated for 30 min in the cuvette before assay (■); thioredoxin-T-(1–73), 1.1×10^{-5} M (×); thioredoxin-T-(74–108), 1.1×10^{-5} M (○); after 3 min the complementing peptide was added to a final concentration of 1.6×10^{-5} M.

Table III: Enzymatic Activity of Thioredoxin-T′ and Thioredoxin Measured as Insulin Disulfide Reduction

thioredoxin-T′ (M)	pH	enzymatic act.[a] $\Delta A_{340} \times$ min^{-1}	enzymatic act.[a] % of thioredoxin
1.3×10^{-5}	6.4	0.035	1.33
1.3×10^{-5}	8.7	0.025	1.00

[a] Thioredoxin (5.4×10^{-7} M) was determined to give an activity of $\Delta A_{340} \times \text{min}^{-1} = 0.110$ at pH 6.4 and of $\Delta A_{340} \times \text{min}^{-1} = 0.105$ at pH 8.7.

was no change in the apparent activity when using 1×10^{-6} or 40×10^{-6} M, which shows that no measurable dissociation of thioredoxin-T′ occurred during the assay.

The lower overall activity of thioredoxin-T′, in the DTNB assay with excess thioredoxin reductase, could be due to a major change in K_m or V_{max} for thioredoxin. For the study of this, a thioredoxin reductase concentration of 1.0×10^{-9} M was used, and the apparent K_m values for thioredoxin-T′ and thioredoxin were determined to be 4.0×10^{-4} M and 4.5×10^{-6} M, respectively. The apparent V_{max} for thioredoxin-T′ was 75% of the value for native thioredoxin. The low activity of thioredoxin-T′ is thus the result of a major increase in the K_m value.

Thioredoxin-T′-$(SH)_2$ was tested as a disulfide reductase by using insulin or ribonucleotide reductase as disulfide substrates. As seen from Table III, thioredoxin-T′-$(SH)_2$ also reduced insulin: the activity relative to thioredoxin was around 1%, and there was no major change in the relative activity at pH 6.4 or 8.6 (Table III). Thioredoxin-T′-$(SH)_2$ was a substrate for *E. coli* ribonucleotide reductase (Table IV). From 3 to 5% of the activity of native thioredoxin-$(SH)_2$ was measured under conditions where the reduction of thioredoxin-T′ was not rate limiting (excess thioredoxin reductase and NADPH).

Table IV: Activity of Thioredoxin-T′-$(SH)_2$ as Hydrogen Donor for *E. coli* Ribonucleotide Reductase[a]

hydrogen donor	concn (M)	act.[b] (nmol of dCDP per 10 min)
thioredoxin-$(SH)_2$	4×10^{-7}	7.1
thioredoxin-T-(1–73)	1.0×10^{-5}	<0.1
thioredoxin-T-(74–108)	1.0×10^{-5}	<0.1
thioredoxin-T′-$(SH)_2$	1.1×10^{-5}	6.1
thioredoxin-T-(1–73) plus thioredoxin-$(SH)_2$	1.0×10^{-5} plus 4×10^{-7}	7.0

[a] The assay was run spectrophotometrically by using a 140-μL final volume with 15 μg of the B1 and the B2 subunits of ribonucleotide reductase (Brown et al., 1969). The concentration of thioredoxin reductase was 5×10^{-7} M and that of NADPH was 0.5 mM. The activity was calculated from the net oxidation of NADPH after addition of 1 mM CDP to the sample cuvette and the control (Brown et al., 1969). [b] The result shows a mean activity for thioredoxin-T′ that was 3.1% of that of thioredoxin.

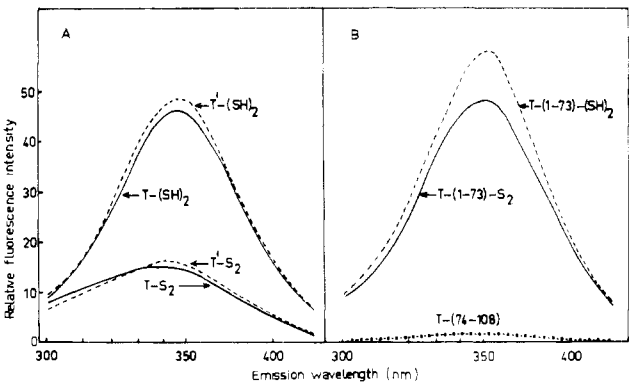

FIGURE 6: (A) Tryptophan fluorescence emission spectra of oxidized thioredoxin (—), reduced thioredoxin (—), oxidized thioredoxin-T′ (- - -), and reduced thioredoxin-T′ (- - -), all at 3.5×10^{-6} M. (B) Tryptophan fluorescence emission spectra of oxidized thioredoxin-T-(1–73) (- - -), reduced thioredoxin-T-(1–73) (—), and thioredoxin-T-(74–108) (●—●), all at 3.5×10^{-6} M.

Kinetics of Generation of Thioredoxin-T′. The formation of active thioredoxin-T′ from a fragment was studied by addition of the complementing fragment to the assay cuvette (Figure 5). There was little activity generated within the first minute by using 1×10^{-5} M of the peptides. The half-time for the generation of activity was about 20 min. These studies were complicated by the observation that the complex generated did not show proportionality in the assay. This was probably due to incomplete complex formation and the presence of nonfunctional inhibitory material (see Figure 2).

Addition of an excess of any of the fragments to thioredoxin-T′ did not give any increase in thioredoxin-T′ activity. This result shows that thioredoxin-T′ is a strong complex with no apparent dissociation in the DTNB assay.

Fluorescence of Thioredoxin-T′. The fluorescence of the tryptophan residues (Trp-28 and Trp-31, Figure 4) of native thioredoxin-S_2 is characterized by a very low quantum yield ($Q = 0.02$) (Stryer et al., 1967; Holmgren, 1972b). Reduction of thioredoxin-S_2 to thioredoxin-$(SH)_2$ is accompanied by a threefold increase in fluorescence at pH 7.0, indicating a localized conformational change (Holmgren, 1972b). The

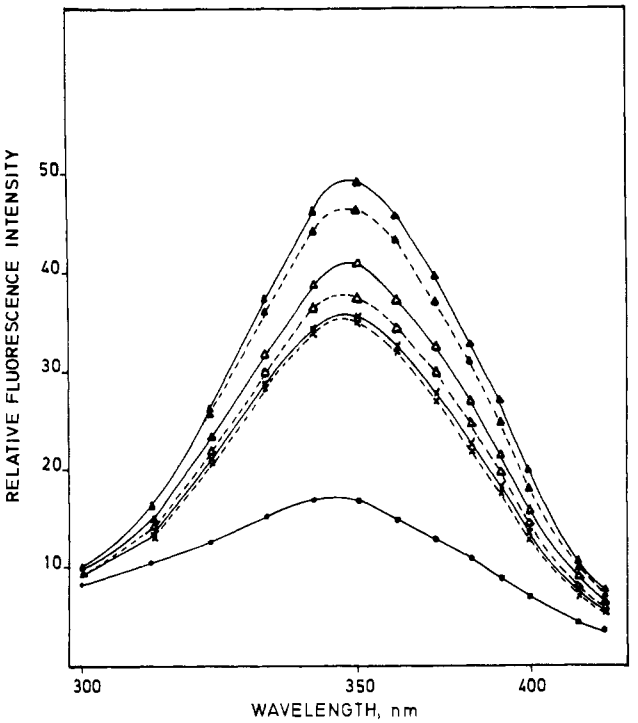

FIGURE 7: Tryptophan fluorescence changes during the formation of thioredoxin-T′. To a cuvette containing oxidized thioredoxin-T-(1–73), 1×10^{-5} M (▲—▲), an equimolar amount of thioredoxin-T-(74–108) was added, and the emission spectrum was determined at 1 min (▲---▲), 30 min (△—△), 60 min (△---△), 100 min (×—×), and 10 h (×---×) after mixing. The emission spectrum of oxidized thioredoxin, 1×10^{-5} M, is also shown (●—●).

fluorescence spectra of thioredoxin-T′-S_2 and thioredoxin-S_2 were very similar (Figure 6A). Reduction of thioredoxin-T′ produced a spectrum that closely resembled that of thioredoxin-$(SH)_2$ with a threefold increase in the quantum yield and a small shift of the wavelength maximum. These spectra were in marked contrast to the spectra of oxidized free peptide thioredoxin-T-(1–73) and peptide-T-(74–108) (Figure 6B). The latter peptide lacks tyrosine and tryptophan residues and has no fluorescence. Thioredoxin-T-(1–73), in its oxidized form, had a 3.5-fold higher quantum yield than native thioredoxin-S_2 and an emission maximum at 355 nm. This is identical with thioredoxin-S_2 in 6 M guanidine hydrochloride (Holmgren, 1972b). Reduction of oxidized thioredoxin-T-(1–73) gave a further 1.2-fold increase in fluorescence.

The change of the tryptophan fluorescence during complementation of thioredoxin-T-(1–73) and thioredoxin-T-(74–108) was determined in the fluorometer (Figure 7). The quenching of the tryptophan fluorescence was a slow process with a half-time of around 30 min at 1×10^{-5} M. The complex formation was apparently incomplete since the fluorescence of the mixture of the fragments was always higher than that of chromatographically purified thioredoxin-T′.

Circular Dichroism of Thioredoxin-T′. Support for the large conformational differences between the free thioredoxin-T-(1–73) and the thioredoxin-T′ complex was obtained from CD spectra (Figure 8). The CD spectra of thioredoxin-T′ and native thioredoxin were very similar with a minimum at 218 ± 2 nm indicating a high content of α helix and β structure. In contrast, the circular dichroism of thioredoxin-T-(1–73) revealed a typical curve of a random-coil structure (Björk & Tanford, 1971).

Radioimmunoassay Studies. The evidence presented above strongly suggested that thioredoxin-T-(1–73) is mostly structureless. Yet, the previous immunochemical experiments

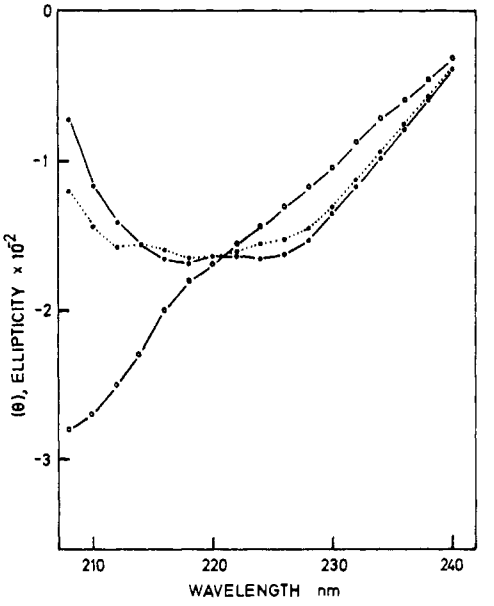

FIGURE 8: Circular dichroism spectra of oxidized thioredoxin, 2.3×10^{-5} M (●—●), oxidized thioredoxin-T′, 1.8×10^{-5} M (●---●), and thioredoxin-T-(1–73), 2.8×10^{-5} M (○—○) at 25 °C.

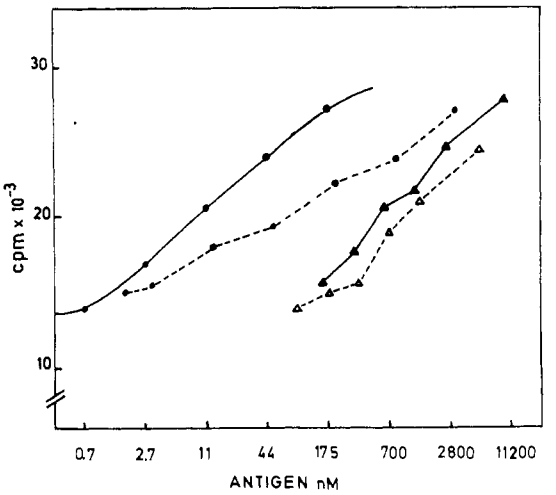

FIGURE 9: Double-antibody radioimmunoassay of native thioredoxin (●—●), thioredoxin-T′ (●---●), thioredoxin-T-(1–73) (▲—▲), and thioredoxin-T-(74–108) (△---△). Unbound [^{125}I]thioredoxin in 100 μL of the supernatant is plotted against log thioredoxin or fragment.

(Slabý & Holmgren, 1975) indicated that both peptide fragments could fold into their "native format" as recognized by antibodies to native thioredoxin. This was further studied by radioimmunoassay. Native thioredoxin gave half-maximal competition with [^{125}I]thioredoxin antibody binding at 3.3×10^{-9} M (Figure 9). The concentrations of half-maximal competition for thioredoxin-T′, thioredoxin-T-(1–73), and thioredoxin-T-(74–108) in assay were 2.7×10^{-8}, 2.1×10^{-7}, and 4.2×10^{-7} M, respectively. These results detect immunochemical conformational differences between thioredoxin-S_2 and thioredoxin-T′ since the competition curves were not parallel. The separate curves for thioredoxin-T-(1–73) and thioredoxin-T-(74–108) are difficult to interpret. The concentration of fragments with conformational similarity to determinants in native thioredoxin was clearly low.

Discussion

The selective cleavage of *E. coli* thioredoxin-S_2 by trypsin at Arg-73 is possible after reversible blocking of all the lysine side chains by citraconic anhydride (Slabý & Holmgren, 1975). This introduces a negative charge in place of the

normally positive charge on the lysine residues. This treatment also denatures thioredoxin to a random coil and makes it very susceptible to the action of trypsin at Arg-73. Native thioredoxin-S_2 is essentially completely resistant to trypsin digestion at room temperature. The generation of thioredoxin-T′ is thus in one way very different from the similar complementing peptide systems obtained from ribonuclease by Richards (1958) and by Anfinsen and collaborators (Anfinsen, 1973) from staphylococcal nuclease. In those cases the two polypeptide chains were selectively cleaved by subtilisin and trypsin, respectively, at residues particularly susceptible to digestion through their location in the protein structures.

The refolding of the separated thioredoxin peptides upon mixing in solution was first observed by the immunochemical behavior of the complex (Slabý & Holmgren, 1975). The three-dimensional structure of thioredoxin shows that Arg-73 is located at the surface of the protein in an accessible loop which separates the COOH-terminal folding unit from the remainder of the molecule. The distance between the active-site S–S bridge and the arginine side chain is about 10 Å. The combined results of the physicochemical and immunochemical measurements show that thioredoxin-T′-S_2 is a stable complex with an overall folding which is very similar to that of native thioredoxin. This is in contrast to the peptide fragments which behave mainly as random coils. Both peptide fragments may have a fairly high conformational equilibrium [K_{conf}; Anfinsen (1973)] and spend a low but significant time in the "native format" conformation they have in thioredoxin-S_2. This is expected from their high secondary structure contents. On the other hand, long-range interactions not present in a separated fragment apparently play important roles in the folding of proteins. This is exemplified by studies of conformational equilibria in helical fragments from myoglobin (Atassi & Singhal, 1970) or fragments from staphylococcal nuclease (Anfinsen, 1973).

The gel chromatographic analysis of thioredoxin-T′ and thioredoxin detects a conformational difference between the structures, consistent with a more flexible structure for thioredoxin-T′. This does not seem to originate from dissociation of the structure into its component peptides but may be the result of a local folding–unfolding reaction close to the arginine residue, where the peptide chain in thioredoxin-T′ has a discontinuity. An obvious structural difference between thioredoxin-T′ and thioredoxin is caused by the two new charges at Arg-73 and Gly-74 and the possibility of increased flexibility of the peptide chain in this region. However, the conformational similarity between thioredoxin and thioredoxin-T′ is shown by the successful crystallization[3] of thioredoxin-T′ under the same conditions as previously used for thioredoxin-S_2 (Holmgren & Söderberg, 1970). Possibly, an X-ray crystallographic study of thioredoxin-T′ would reveal the conformational differences to native thioredoxin-S_2.

The enzymatic activity of thioredoxin-T′-S_2 as a substrate for thioredoxin reductase is surprisingly low in view of its stability and conformational similarity to native thioredoxin-S_2. The main effect is an increase of the K_m value around 2 orders of magnitude, which indicates that the binding to the enzyme is affected in a major way. It is possible that the artificial charge at Arg-73 close to the S–S bridge interferes with critical electrostatistical binding or orienting interactions with the enzyme (Holmgren & Slabý, 1979). The reduced form of thioredoxin-T′ is functional as a disulfide reductase with insulin or ribonucleotide reductase disulfides. The refolded thioredoxin-T′ is thus able to undergo the localized conformational change that accompanies the oxidation–reduction of the disulfide bridge at the active site. This is in agreement with the changes in fluorescence spectra observed for thioredoxin-T′-S_2 upon reduction.

The results of the enzymatic activity determinations and fluorescence spectra recordings detect no evidence for dissociation of thioredoxin-T′ at 1.0×10^{-6} M concentration. This is supported by the electrophoresis and gel chromatography results. The results are compatible with a dissociation constant for the complex lower than 10^{-8} M. The formation of the complex is a surprisingly slow process as observed with either the generation of enzyme activity or the quenching of the tryptophan fluorescence in the thioredoxin-T′. Two models may account for this result: (1) the rapid formation of an initially disordered complex which then is slowly converted into the thioredoxin-like folded molecule or (2) a binding of two "native format" prefolded fragments corresponding to the approximate conformations of the fragments in native thioredoxin-S_2. The binding may be slow because the conformational equilibrium is unfavorable, making the effective concentrations of the fragments very low. It should be possible to distinguish between these alternatives by further kinetic experiments. It seems essential, then, to have peptide fragments which are functionally more homogeneous. This is possible by avoiding separation of the fragments in 50% acetic acid and splitting refolded thioredoxin-T′ in guanidine hydrochloride.[3]

The tryptophan fluorescence studies described here show the usefulness of this technique to follow conformational changes in thioredoxin. From previous studies, the disulfide bridge at the active site was suggested as an important quencher of both tryptophan residues in thioredoxin-S_2 (Holmgren, 1972b). The three-dimensional structure of thioredoxin-S_2 shows that Trp-31 is fully exposed to solvent (Holmgren et al., 1975). Trp-28 is located in a minipocket, and chemical modification [Holmgren (1973) and footnote 4] shows that this residue is responsible for almost all of the increase in tryptophan fluorescence upon nonenzymatic reduction of thioredoxin-S_2. Nuclear magnetic resonance studies (Holmgren & Roberts, 1976) also show the nonequivalence of the two tryptophan residues in thioredoxin.

Thioredoxin from *E. coli* may now be analyzed by genetic techniques since the location of the gene called *TrxA* has been mapped in *E. coli* K12 (Mark et al., 1977). The essential function of thioredoxin as a subunit of the T7 virus DNA polymerase (Mark & Richardson, 1976) allows the isolation of *E. coli* mutants of the protein with deficient thioredoxin (Chamberlin, 1974). We have recently analyzed thioredoxin from one such mutant (tsnC 7007)[5] which has Gly-92 exchanged with an Asp residue. The possibility of applying hybrid DNA techniques and the use of defined amber suppressor strains should make structural studies of the folding and function of thioredoxin and thioredoxin-T′ very fruitful.

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Thioredoxin-C′: Mechanism of Noncovalent Complementation and Reactions of the Refolded Complex and the Active Site Containing Fragment with Thioredoxin Reductase†

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ABSTRACT: Thioredoxin-C′ is a refolded complex of the two inactive fragments thioredoxin-C-(1–37) and thioredoxin-C-(38–108) obtained by CNBr cleavage at the single Met-37 of thioredoxin from *Escherichia coli*. Thioredoxin-C′-S_2 is a weak complex that dissociates during gel chromatography, during gel electrophoresis, or in activity measurements as a substrate for NADPH and thioredoxin reductase. The association of the peptide fragments to thioredoxin-C′-S_2 and its dissociation were both rapid processes with half-times of 1–2 min at 10^{-6} M. Variation of the relative proportion of the fragments in the assay of thioredoxin-C′ with thioredoxin reductase gave an apparent K_D for thioredoxin-C′-S_2 of 2×10^{-6} M at 25 °C. Thioredoxin-C′-S_2 was a good substrate for NADPH and thioredoxin reductase and was calculated to have more than 50% relative activity when compared with thioredoxin-S_2. In contrast, thioredoxin-C′-$(SH)_2$ was inactive as a hydrogen donor for *E. coli* ribonucleotide reductase or as an insulin disulfide reductase, strongly suggesting that Met-37 is essential for the conformational change on oxidoreduction of thioredoxin. The active-site disulfide in thioredoxin-C-(1–37) was not reduced by NADPH and thioredoxin reductase; instead, the fragment was an inhibitor of thioredoxin reductase in the presence of NADPH and DTNB, which suggested nucleated folding of the fragment to generate a binding site for thioredoxin reductase. Structure–function relationships for chemically modified thioredoxin-C-(1–37) suggest essential functions for Met-37 and Lys-36 of thioredoxin in the interactions with thioredoxin reductase and ribonucleotide reductase. A model, based on the results with thioredoxin-C′, is presented to describe the three-dimensional complementarity of thioredoxin and thioredoxin reductase.

The three-dimensional structure of the oxidized form of *Escherichia coli* thioredoxin (thioredoxin-S_2)[1] has a high content of secondary structure that includes a central core of five strands of β-pleated sheet surrounded by four α helices (Figure 1) (Holmgren et al., 1975). The residues involved in the active center 14-membered disulfide bridge of the molecule form a protrusion between one of the helices and the middle strand of the pleated sheet. Thioredoxin has only one residue each of methionine (Met-37) and arginine (Arg-73) in its polypeptide chain of 108 amino acid residues. This has enabled

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[1] Abbreviations used: thioredoxin-S_2 (T-S_2) and thioredoxin-$(SH)_2$ [T-$(SH)_2$], the oxidized and reduced forms of thioredoxin, respectively; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid). Peptide fragments have been designed by an adoption of the rules of the IUPAC–IUB Commission on Biochemical Nomenclature. Fragments obtained after CNBr cleavage are denoted by C and after selective cleavage with trypsin are denoted by T. The reconstituted noncovalent complexes are denoted thioredoxin-C′ and thioredoxin-T′ (Slabý & Holmgren, 1975).

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