THIOREDOXIN-C′: RECONSTITUTION OF AN ACTIVE FORM OF *ESCHERICHIA COLI* THIOREDOXIN FROM TWO NONCOVALENTLY LINKED CYANOGEN BROMIDE PEPTIDE FRAGMENTS

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1. Introduction

It was previously shown that treatment of the small electron transport protein thioredoxin from *Escherichia coli* with CNBr resulted in cleavage of the peptide bond following the single methionine residue (Met-37) [1]. Two peptide fragments of 37 and 71 residues, previously denoted peptide B and A, respectively, were isolated after chromatography on Sephadex G-50 in 50% CH_3COOH [1]. The elucidation of the amino acid sequence of the two fragments [2–4] led to the complete primary structure of thioredoxin [5], which has a single oxidation–reduction active disulfide bridge formed from two half-cystine residues (Cys-32 and Cys-35).

Thioredoxin-C-(1-37) [6], the N-terminal fragment, contained the oxidation–reduction active disulfide bridge of thioredoxin but was not reducible by NADPH and thioredoxin reductase [1]. In this paper will be summarized experiments which show that upon mixing thioredoxin-C-(1-37) with thioredoxin-C-(38-108) a spontaneous reconstitution occurs to give a complex called thioredoxin-C′ which has enzymatic activity with NADPH and thioredoxin reductase. Thioredoxin-C′ can be isolated by gel chromatography and has immunochemical properties similar to native thioredoxin. The results suggest that thioredoxin-C′ has a structure similar to native thioredoxin and is formed as a result of the cooperative interactions normally stabilizing the tertiary structure of the protein.

2. Materials and methods

Thioredoxin was purified from *E. coli* B with "Method 2" as described previously [1]. Preparations used in this study were homogeneous and contained 73 nmoles of thioredoxin per A_{280} unit [1]. Cleavage of thioredoxin with CNBr and separation of the mixture by chromatography on Sephadex G-50 in 50% CH_3COOH were performed as described [1]. The isolated peptides were lyophilized and dissolved in 0.06 M NH_4HCO_3, pH 8.00 at a concentration around 1×10^{-3} M. Concentrations of peptide fragments were determined by measuring the absorbance at 280 nm (A_{280}) using molar extinction coefficients of 10,000 liters M^{-1} cm^{-1} for thioredoxin-C-(1-37) and 3,700 liters M^{-1} cm^{-1} for thioredoxin-C-(38-108) [1].

Thioredoxin reductase from *E. coli* B after Sephadex G-100 chromatography [7] was a kind gift of Dr. Lars Thelander. NADPH and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were products of Sigma Chemical Company. Bovine serum albumin was from British Drug Houses. Rabbit antisera against thioredoxin from *E. coli* were obtained as previously described [8]. Sephadex G-50 was from Pharmacia Fine Chemicals. All other chemicals were of highest purity commercially available.

2.1. *Immunochemical methods*

Quantitative immunoprecipitin tests were performed by incubating 50 µl of rabbit antithioredoxin antiserum with increasing amounts of antigen

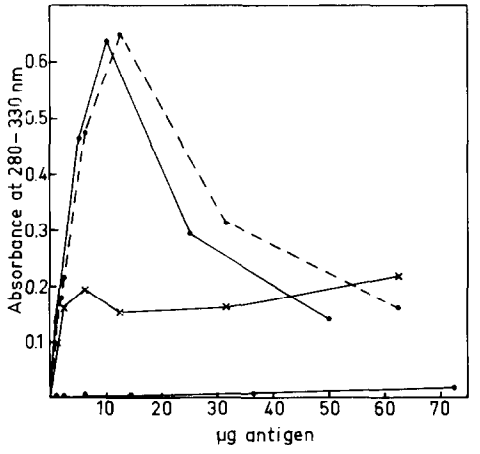

Fig. 1. Quantitative immunoprecipitin reaction of native thioredoxin (○—○—○); thioredoxin-C-(137) (●—●—●); thioredoxin-C-(38-108) (X—X—X); and a 1 : 1 mixture of thioredoxin-C-(1-37) and thioredoxin-C-(38-108) (● - - ● - - ●). For experimental details see Materials and methods.

in a final volume of 200 µl of 0.15 M NaCl–0.01 M potassium phosphate, pH 7.0. After incubation for 1 hr at 37° and 18 hr at +4° precipitates were collected by centrifugation and washed three times with ice-cold 0.15 M NaCl. Finally the washed precipitates were dissolved in 0.500 ml of 0.10 M NaOH and the absorbance at 280 nm and 330 nm determined in a Zeiss PMQ II spectrophotometer.

2.2. *Enzymatic assay of thioredoxin*

The DTNB-method was used [9]. Reduced thioredoxin formed enzymatically by NADPH and thioredoxin reductase is reoxidized by DTNB forming the coloured 3-carboxy-4-nitrothiophenolate ion. Each of two cuvettes contained in a volume of 520 µl: 60 µmoles of Tris-HCl buffer, pH 8.0; 5 µmoles of EDTA; 0.10 µmole of NADPH; 1.0 mg of bovine serum albumin; 0.100 µmole of DTNB and 2% (v/v) of ethanol. To one cuvette was added thioredoxin or peptide fragments and the other cuvette served as blank. The reaction was started by addition of thioredoxin reductase (4 µg) to both cuvettes and followed by measuring the absorbance at 412 nm in a Zeiss PMQ II spectrophotometer.

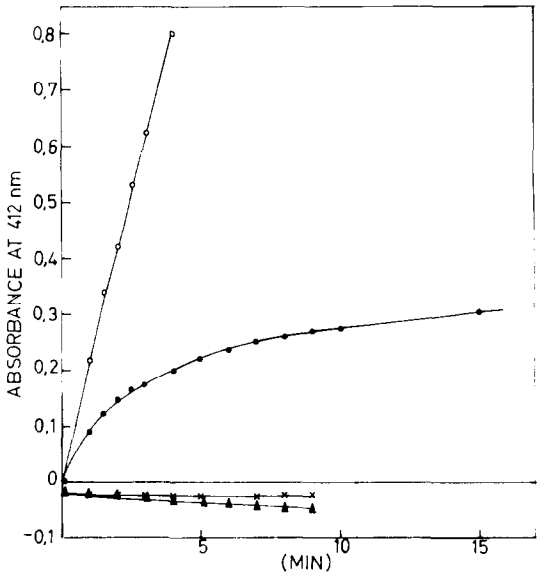

Fig. 2. Enzymatic activity of thioredoxin-C′ and peptide fragments. Activity of native thioredoxin 2×10^{-6} M (○—○—○); thioredoxin-C-(1-37) 4×10^{-6} M (▲—▲—▲); thioredoxin-C-(38-108) 4×10^{-6} M (X—X—X); and thioredoxin-C-(1-37) + thioredoxin-C-(38-108) = thioredoxin-C′ 3×10^{-6} M (●—●—●).

3. Results and discussion

Recently potent precipitating antisera against native oxidized *E. coli* thioredoxin were obtained from immunized rabbits [8]. A quantitative immunoprecipitin curve for thioredoxin and the two cyanogen bromide fragments of the protein with an antiserum is shown in fig. 1. Native thioredoxin had the characteristics of a complete antigen [10] with a zone of inhibition at high antigen concentrations. Thioredoxin-C-(1-37) gave essentially no precipitate whereas thioredoxin-C-(38-108) gave about 25% of the precipitate obtained with a corresponding amount of native thioredoxin. Thioredoxin-C-(38-108) however gave no zone of inhibition and therefore had the character of an incomplete antigen [10]. A mixture of thioredoxin-C-(1-37) and thioredoxin-C-(38-108) in equimolar amounts at 1 mg per ml however showed an immunoprecipitin curve (fig. 1) which was almost superimposable on the curve for native thioredoxin. This result indicated that a complex called thioredoxin-C′ was formed with an immunochemical structure similar to native thioredoxin.

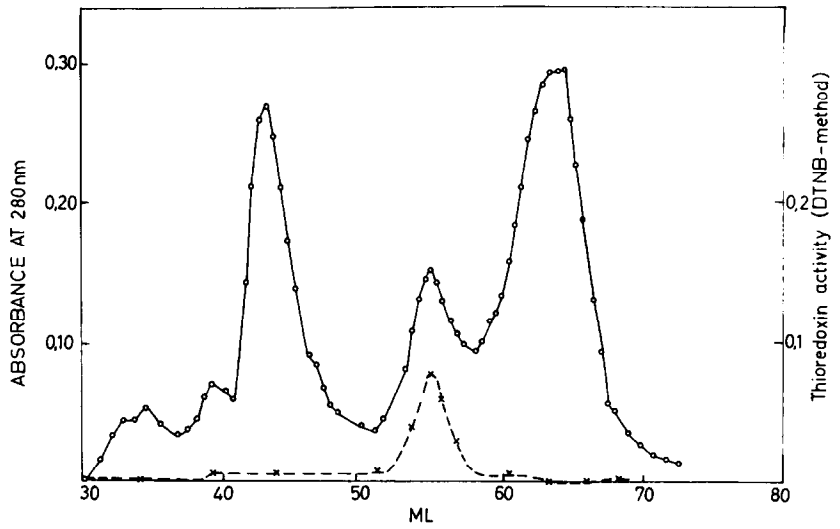

Fig. 3. Chromatography of a mixture of 300 μl of thioredoxin-C-(1-37) 1.5 × 10^{-3} M and 300 μl thioredoxin-C-(38-108) 1.25 × 10^{-3} M on a column of Sephadex G-50 (0.9 × 140 cm). The column was equilibrated with 0.15 M NaCl–0.01 M potassium phosphate, pH 7.0. Absorbance at 280 nm (○—○—○). Enzymatic activity (×—×—×) was determined on 10 μl aliquots from the fractions by the DTNB-method.

The reconstitution of full antigenic activity of thioredoxin in thioredoxin-C′ prompted an investigation of the enzymatic activity of thioredoxin-C′. As shown in fig. 2, thioredoxin-C′ at 3 × 10^{-6} M had 20% of the initial activity of a corresponding amount of native thioredoxin in a DTNB-assay. The isolated peptide-C-(1-37) and peptide-C-(38-108) were both inactive, in agreement with earlier results which showed that the disulfide of thioredoxin-C-(1-37) was not reducible by thioredoxin reductase [1]. Thioredoxin-C′ was thus a substrate for thioredoxin reductase in its oxidized form and could be reoxidized by DTNB similarly to native thioredoxin since it catalyzed reduction of DTNB (fig. 2).

The activity of thioredoxin-C′ decreased with time in the DTNB-assay (fig. 2). The mechanism behind this decrease so far remains unclear. However, the possibility that free reduced thioredoxin-C-(1-37) in reversible equilibrium with thioredoxin-C′ is irreversibly inactivated by DTNB should be considered. Thioredoxin-C-(1-37) is presumed to be structureless in water [1, 11] and whether oxidation of the two thiol groups of the reduced form results in quantitative oxidation to a disulfide as in native thioredoxin remains to be determined. Another possible explanation of the decrease in activity is inactivation of thioredoxin reductase by thioredoxin-C-(1-37) which has been observed in the presence of NADPH and DTNB [12].

The assay of thioredoxin-C′ (fig. 2) was performed at a concentration of 3 × 10^{-6} M with an excess of thioredoxin reductase. The K_m value of native thioredoxin for thioredoxin reductase is 5 × 10^{-6} M [13]. The dissociation constant for thioredoxin-C′ can be expected to be in the range of 10^{-5}–10^{-6} M if the peptide–peptide interaction is of similar strength to that recently estimated for ribonuclease-S′ [14, 15]. Further studies are thus necessary to quantitate the activity of thioredoxin-C′ as a substrate for thioredoxin reductase since multiple equilibrium processes are involved.

Direct evidence of reconstitution of thioredoxin from thioredoxin-C-(1-37) and thioredoxin-C-(38-108) was obtained by the gel chromatography experiment shown in fig. 3. The mixture of thioredoxin-C-(1-37) and thioredoxin-C-(38-108) was chromatographed on a column of Sephadex G-50 at pH 7.0. One main peak of enzymatic activity with thioredoxin reductase was observed. This peak, which corresponded to about 20% of the applied material, had

a K_d value of 0.5 identical with that of native oxidized thioredoxin. The first large peak with a low enzymatic activity appeared to be a dimer of thioredoxin-C′ whereas the third main peak contained some uncomplexed thioredoxin-C-(1-37) identified by its high A_{280} absorbance due to its content of two tryptophan residues. Chromatography of thioredoxin-C-(1-37) and thioredoxin-C-(38-108) separately each gave a single symmetrical peak [12].

The results described above show that the two CNBr-peptide fragments of thioredoxin from *E. coli* can be reconstituted to a molecule thioredoxin-C′ which has considerable enzymatic activity with thioredoxin reductase. Further studies are needed for a more detailed characterization of the properties of thioredoxin-C′ and the conditions for its formation. The reconstitution of thioredoxin-C′ should present many useful applications in a direct examination of side chain interactions underlying the formation of the three dimensional structure of thioredoxin as well as of relationships between structure and function, much as in the case of bovine pancreatic ribonuclease-S [16, 17] and staphylococcal nuclease-T [18, 19].

Acknowledgements

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