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Purification and Properties of *Escherichia coli*-*Corynebacterium nephridii* Hybrid Thioredoxin

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Abstract: In earlier studies, the genes encoding *Escherichia coli* thioredoxin and *Corynebacterium nephridii* thioredoxin C-3 were fused via a common restriction site in the nucleotide sequence coding for the active site of the proteins to generate two chimeric thioredoxins, designated E-C3 (N to C-terminal) and C3-E. The hybrid thioredoxins were overexpressed in *E. coli* from the cloned chimeric thioredoxin genes by a T7 promoter/polymerase system. To investigate the structure-function relationship of thioredoxin, we purified the E-C3 hybrid thioredoxin through ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sephadex G-50 gel filtration. Its purity was examined on SDS-polyacrylamide gel electrophoresis and the molecular weight of the purified E-C3 hybrid thioredoxin was estimated to be 12,000. On native polyacrylamide gels, the purified E-C3 hybrid thioredoxin shows a much lower mobility than *E. coli* thioredoxin. E-C3 hybrid thioredoxin exhibits a 40-fold lower catalytic efficiency with *E. coli* thioredoxin reductase than *E. coli* thioredoxin. It was shown to catalyze the reduction of insulin disulfide by dithiothreitol. The purified E-C3 hybrid thioredoxin was also characterized in other aspects.

Key words: *Corynebacterium nephridii*, *Escherichia coli*, thioredoxin,

Thioredoxins have been isolated from many organisms and found to participate in a variety of biological reactions. They act as hydrogen donors for various reductive enzymes, protein disulfide oxidoreductases, photosynthetic regulatory factors, one subunit of T7 DNA polymerase, essential components for the assembly of small viruses, and possibly protein disulfide isomerases (Holmgren, 1985; Holmgren, 1989). Thioredoxin is also an activating factor for the cytosolic glucocorticoid receptor (Grippo *et al.*, 1983) and the extracellular domain of the interferon-γ receptor (Fountoulakis, 1992). *E. coli* thioredoxin has been reported to be an activator of the Ref-1 nuclear protein that modulates DNA binding of the AP-1 transcription factor (Abate *et al.*, 1990). Thioredoxin also modulates the DNA binding of other transcription factors (Cromlish and Roeder, 1989; Peleg *et al.*, 1989) including the cytoplasmic transcription factor NF-κB, which is a member of the rel protooncogene family (Schreck *et al.*, 1991). Furthermore, thioredoxin can reduce H_2O_2 and scavenge free radicals (Schallreuter and Wood, 1986; Spector *et al.*, 1988).

The amino acid sequence of *E. coli* thioredoxin (Holmgren, 1968) and the nucleotide sequence of the corresponding *trxA* gene (Lim *et al.*, 1985) are known. *E. coli* thioredoxin contains 108 amino acid residues with the active site Cys-Gly-Pro-Cys forming a disulfide bond in the oxidized form (Holmgren, 1985). Oxidized thioredoxin (Trx-S_2) is reduced to Trx-$(SH)_2$ by a NADPH-dependent thioredoxin reductase. The three dimensional structure of Trx-S_2 has been solved by X-ray crystallography and refined to a 1.7-Å resolution (Katti *et al.*, 1990), and the NMR solution structure of Trx-$(SH)_2$ has been calculated and refined using distance geometry and molecular dynamics (Dyson *et al.*, 1990; Jeng *et al.*, 1994; Jeng and Dyson, 1995). According to the X-ray crystallography structure, *E. coli* thioredoxin is composed of two domains. A thioredoxin (thioredoxin C-1) from the gram-negative bacterium *Corynebacterium nephridii* was purified to homogeneity in 1981 (Meng and Hogenkamp, 1981). A gene encoding a second thioredoxin (thioredoxin C-2) from *C. nephridii* was isolated and sequenced (Lim *et al.*, 1987), and purified (McFarlan *et al.*, 1989). A third thioredoxin (thioredoxin C-3) from *C. nephridii* was also isolated and sequenced (Lim *et al.*, 1995). It was found to contain 145 amino acid residues predicted from the nucleotide sequence and it contains 6 cysteine residues, two of which reside in the known active center sequence.

In previous studies we constructed and characterized two reciprocal hybrid thioredoxin genes (E-C3 and C3-

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E) between *E. coli* and *Corynebacterium* C-3 thioredoxin genes using a common *Ava*II site (Sa *et al.*, 1995). In this paper we present the purification and properties of *E. coli-Corynebacterium* C-3 hybrid thioredoxin (E-C3) from an *E. coli* strain harboring the overexpressed E-C3 hybrid thioredoxin gene.

Materials and Methods

Materials

Restriction endonucleases (*Eco*RI and *Hin*dIII) and T4 DNA ligase were purchased from New England Biolabs. Agarose, ampicillin, and kanamycin were obtained from the Sigma Chemical Company. 5,5'-dithio-2-nitrobenzoic acid (DTNB), NADPH, rifampicin, insulin, acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), and ammonium persulfate were also obtained from the Sigma Chemical Company. DEAE-cellulose and Sephadex G-50 were the products of Whatman and the Sigma Chemical Company, respectively. L-^{35}S-Methionine (1000 Ci/mmol) was purchased from the Amersham Corporation. *E. coli* thioredoxin reductase was a kind gift from J. A. Fuchs, Department of Biochemistry, University of Minnesota.

Bacterial strains and plasmids

E. coli strain BH2012 (F$^-$, *araD139* ?, *galU*, *galK*, *hsr*, *rpsL*, *metA46*, *argH1*, *trxA7004*, *ilvC::Tn5*) was used as a recipient for transformation. Strain BH5262 (F$^-$, *araD139* ?, *galU*, *galK*, *hsr*, *rpsL*, *argH1*, *trxA7004*, *gshA*, *srl::Tn10*) was used as a recipient for co-transformation with plasmid pGP1-2 and a recombinant plasmid harboring the E-C3 hybrid thioredoxin gene under a T7 promoter. *E. coli* strains were grown in Luria-Bertani medium (Miller, 1972). When needed, ampicillin and kanamycin were added at final concentrations of 50 and 40 μg/ml, respectively.

Plasmid pJS30 is a pTZ18R derivative containing a hybrid thioredoxin gene encoding E-C3 hybrid thioredoxin, whereas plasmid pJS40 contains a C3-E hybrid thioredoxin gene (Sa *et al.*, 1995). The two pTZ18R derivatives were digested with *Eco*RI and *Hin*dIII. The EcoRI-*Hin*dIII fragments were inserted into the *Eco*RI-*Hin*dIII linker region of plasmid pT7-5. In plasmid pT7-5 the gene encoding β-lactamase is in opposite orientation with T7 promoter, and expression by T7 RNA polymerase is limited exclusively to cloned genes (Tabor and Richardson, 1985). The structures of the resulting plasmids encoding hybrid thioredoxins were confirmed by double digestion with *Eco*RI and *Hin*dIII (data not shown). The two pT7-5 derivatives were deginated pJS100 and pJS200, which encoded E-C3 and C3-E hybrid thioredoxins, respectively.

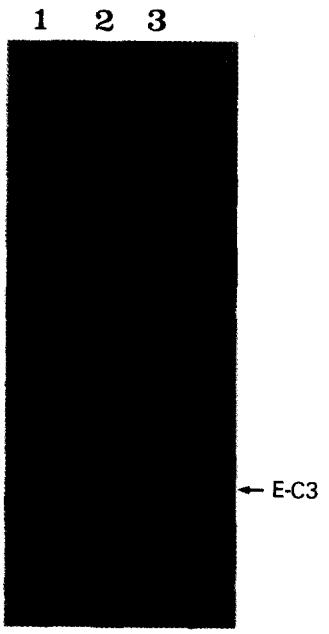

Fig. 1. Identification of two hybrid thioredoxin overproduced by T7 RNA polymerase/promoter system. Plasmid pJS100 carries the E-C3 hybrid thioredoxin gene producing E-C3 hybrid thioredoxin indicated by an arrow, whereas pJS200 harbors C3-E hybrid thioredoxin gene under T7 promoter. Lane 1 : BH5262/pGP1-2/pT7-5; Lane 2 : BH5262/pGP1-2/pJS100; Lane 3 : BH5262/pGP1-2/pJS200.

Labeling of the E-C3 hybrid thioredoxin using the T7 RNA polymerase/promoter system

The two pT7-5 derivatives, pJS100 and pJS200, were introduced into thioredoxin-deficient strain BH 5262 together with plasmid pGP1-2, which carried a gene encoding T7 RNA polymerase. Their expression products were specifically labelled with ^{35}S-methionine by the T7 RNA polymerase/promoter system (Tabor and Richardson, 1985). An autoradiographic pattern of labelled proteins showed the production of two hybrid thioredoxins (Fig. 1). Strain BH5262/pGP1-2/pJS 100 produced the E-C3 hybrid thioredoxin as indicated by an arrow (Fig. 1, lane 2). The E-C3 hybrid thioredoxin was traced by radioactivity instead of a thioredoxin assay during purification.

Overproduction of the E-C3 hybrid thioredoxin

Strain BH5262/pGP1-2/pJS100 was grown in a large-scale LB broth with ampicillin and kanamycin at 30℃ up to an OD_{600} of 1.5. After the temperature was shifted to 42℃, the cells were incubated for 30 min, and rifampicin (final concentration, 100 μg/ml) was added in order to inhibit *E. coli* RNA polymerase. The temperature was shifted to 37℃ for an additional two hours, and then the cells were harvested (Tabor and Richardson, 1985).

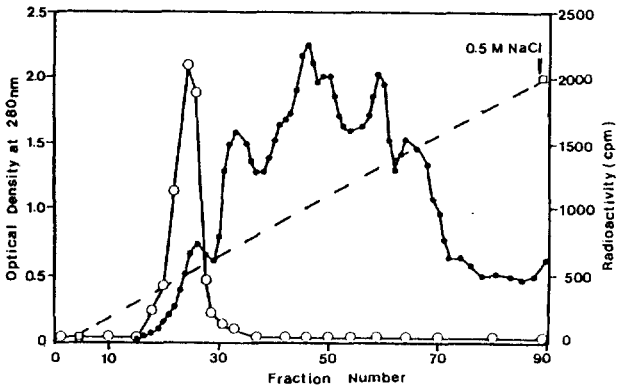

Fig. 2. Elution profile of E-C3 hybrid thioredoxin from DE-52 column chromatography. The radioactive fractions were obtained from the 40~60% ammonium sulfate fractionations, and then loaded onto a column of DEAE-52 column (2.5×15 cm) equilibrated in 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The column was developed with a linear gradient of 0~5 N NaCl in the equilibration buffer. The fractions were collected and determined for absorbance at 280 nm (●–●) and radioactivity (○–○).

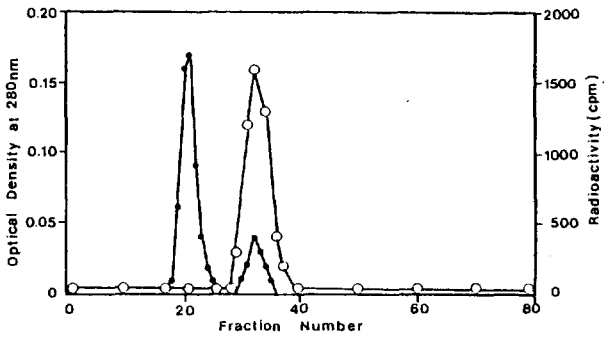

Fig. 3. Sephadex G-50 chromatography of E-C3 hybrid thioredoxin. The radioactive fractions were obtained from the DEAE-cellulose columns, and then loaded onto a column of sephadex G-50 (2.8×43 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA. The column was developed in equilibration buffer. Fractions were collected, and determined for absorbance at 280 nm (●–●) and radioactivity (○–○).

Preparation of crude extracts

A twenty liter culture of BH5262/pGP1-2/pJS100 was prepared. Using the T7 RNA polymerase/promoter system described above, the E-C3 hybrid thioredoxin gene was selectively overexpressed. Cells were harvested and washed twice in 10% sucrose-50 mM Tris-HCl buffer (pH 7.5). The yield from a 20 L culture was about 42 g by wet weight. Separately, the only plasmid-encoded protein, E-C3 hybrid thioredoxin, was specifically labelled with ^{35}S-methionine in a small-scale 100 ml culture. Cell pellets obtained after mixing the small-scale labelled culture with large-scale culture cells were resuspended in 200 ml of 10% sucrose-50 mM Tris-HCl buffer (pH 7.5) and disrupted by lysozyme treatment at a final concentration of 0.2 mg per ml, and sonication. The suspension was centrifuged at 15,000 rpm for one hour. The supernatant fluid (crude extract) had a volume of 200 ml.

Ammonium sulfate fractionation

The crude extract was fractionated with 40~60% ammonium sulfate. The precipitate was centrifuged for 30 min at 15,000 rpm in the Sorvall SS-34 rotor, and the pellets were redissolved in buffer A [50 mM Tris-HCl (pH 7.5), 1 mM EDTA]. The suspension was dialyzed against two changes of 2 L buffer A.

DEAE-cellulose chromatography

The dialyzed sample was applied to a DEAE-cellulose column (2.5×15 cm) equilibrated with buffer A. Elution was carried out with a linear gradient of 0~0.5 N NaCl in buffer A. The radioactive fractions were pooled. A typical elution profile of DEAE-52 column chromatography was shown in Fig. 2.

Sephadex G-50 gel filtration

The radioactive fractions, obtained from the DEAE-cellulose column, were precipitated using 60% ammonium sulfate. After centrifugation, the precipitate was dissolved in buffer A, and then applied to a Sephadex G-50 column (2.8×43 cm). The column was eluted with the same buffer and radioactive fractions were pooled. An elution profile of Sephadex G-50 chromatography is shown in Fig. 3.

Enzyme assays

Two methods were used to detect and quantitate thioredoxin.

Method 1 : Thioredoxin was assayed with thioredoxin reductase by using DTNB as an electron acceptor (Holmgren, 1977; Luthman and Holmgren, 1982). This method was used for kinetic analysis.

Method 2 : Thioredoxin-catalyzed reduction of insulin by DTT was monitored as a turbidity increase at 650 nm (Holmgren, 1979). This method was used to estimate the ability of the hybrid thioredoxin to function as a protein disulfide reductase.

Protein determination

Protein concentration was estimated by the method of Lowry *et al.* (1951). The protein content in chromatography fractions was determined by absorbance measurements at 280 nm.

Electrophoresis

Native and SDS slab gels (16×18 cm) containing 12% acrylamide were prepared and run at room temperature in 0.1 M Tris/glycine buffer (pH 8.3) (Laemmli, 1970). Proteins were stained with Coomassie bril-

Table 1. Purification of E-C3 hybrid thioredoxin from *Escherichia coli*

Step	Total proteins (mg)	Total activity[a] (ΔA_{412}/min)	Specific activity[b] (ΔA_{412}/min/mg)
Crude extract	740	7.60	0.010
Ammonium sulfate precipitation	127	2.40	0.020
DE-52 chromatography	23	1.87	0.081
Sephadex G-50 chromatography	1.4	0.62	0.443

[a]Enzyme activity was expressed as the change in absorbance at 412 nm per min.
[b]Specific activity equals to enzyme activity per mg protein.

liant blue R-250.

Results and Discussion

E. coli thioredoxin has been frequently used as a model system in studies on protein structure and function. In previous studies two reciprocal hybrid thioredoxin genes between *E. coli* and *Corynebacterium* were constructed and characterized (Sa *et al.*, 1995). The two hybrid thioredoxins complemented phenotypes of a thioredoxin-deficient *E. coli* strain. A strain containing the C3-E hybrid thioredoxin supported growth of the T7 phage, whereas a strain expressing the E-C3 hybrid thioredoxin did not. Both hybrids supported growth of M13 phages. The extract from the *E. coli* thioredoxin-deficient strain harboring the E-C3 hybrid thioredoxin gene showed a much reduced catalytic efficiency in the DTNB assay compared with the extract from the same *E. coli* strain containing the *E. coli* thioredoxin gene. The difference in catalytic efficiency reflects either a low affinity of the E-C3 hybrid thioredoxin for *E. coli* thioredoxin reductase, or a relatively unstable character of the E-C3 hybrid thioredoxin.

To characterize the E-C3 hybrid thioredoxin, we purified and characterized the E-C3 hybrid thioredoxin from a thioredoxin-deficient *E. coli* strain containing a plasmid encoding E-C3 hybrid thioredoxin. The E-C3 hybrid thioredoxin gene was cloned into the plasmid pT7-5. Transformants were identified by complementation of a thioredoxin-negative strain BH2012 and the structures of recombinant plasmids were confirmed by restriction mapping (Data not shown). The E-C3 hybrid thioredoxin was labeled using a T7 RNA polymerase/promoter system as described in 'Materials and Methods'. In the autoradiographic pattern (Fig. 1), plasmid pJS100 gave the production of the E-C3 hybrid thioredoxin. The mRNA produced from the *E. coli* thioredoxin gene contained two potential translational initiation sites, one of which could initiate the synthesis of a protein 19 amino acids longer (called extended thioredoxin, M.W., 15,000 daltons) than the *E. coli* thioredoxin of 108 amino acid reisdues (M.W., 12,000 daltons) (Lim *et al.*, 1985, 1991, 1992; Sa *et al.*, 1995). Plasmid pJS100 coded for E-C3 hybrid thioredoxin having the N-terminal sequence from *E. coli* thioredoxin and the C-terminal sequence from *C. nephridii* thioredoxin C3. Thus, strain BH5262/pGP1-2/pJS100 produced two proteins with molecular weights of 15,000 and 12,000 daltons, similar to the *E. coli* thioredoxin gene products (Fig. 1, lane 2). The E-C3 hybrid thioredoxin (M.W., 12,000 daltons) was traced by radioactivity. Twenty liter cultured cells of BH5262/pGP 1-2/pJS100 and 100 ml small-scale labelled cultures were mixed and resuspended as described in 'Materials and Methods'. The crude extract was fractionated with 40~60% ammonium sulfate and the supernatant was obtained. The E-C3 hybrid thioredoxin was purified from the supernatant by DEAE-cellulose chromatography using 0~0.5 N NaCl gradient (Fig. 2). Radioactive fractions were pooled and separated by Sephadex G-50 chromatography (Fig. 3).

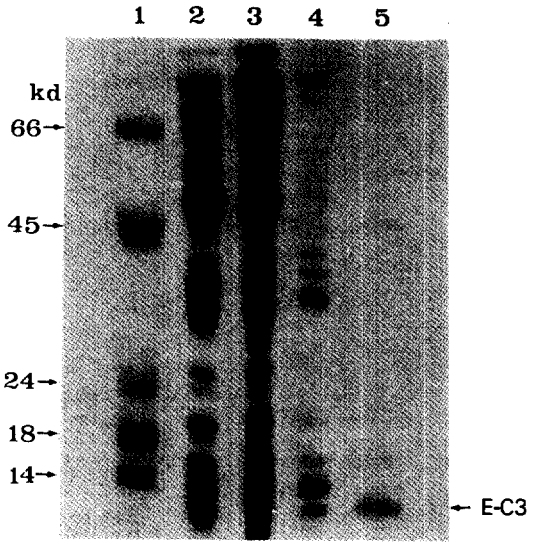

Fig. 4. SDS-polyacrylamide gel electrophoretic pattern of each fractions obtained during purification process. SDS-PAGE was performed in 12% polyacrylamide gel by the method of Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250. Lane 1 : Protein markers; Lane 2 : Crude extract; Lane 3 : 40~60 % Ammonium sulfate fractionation; Lane 4 : DE-52 Cellulose chromatography; Lane 5 : Sephadex G-50 Gel filtration.

Table 1 summarizes the purification scheme on E-C3 hybrid thioredoxin. The final preparation was purified approximately 44-fold. Purity of the protein was confirmed by SDS-polyacrylamide gel electrophoresis (Fig. 4). From the electrophoretic pattern, the Sephadex G-50 gel filtration fraction showing a single band con-

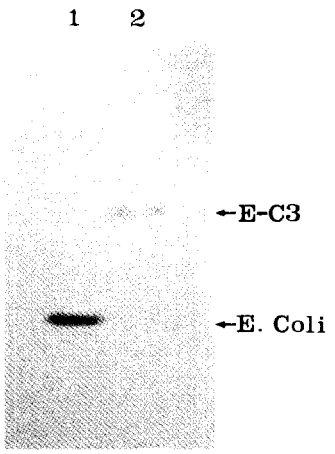

Fig. 5. Separation of thioredoxins by native polyacrylamide gel electrophoresis. Protein of each type was loaded onto a native 12% polyacrylamide gel and run at pH 8.3. Lane 1 : *E. coli* thioredoxin; Lane 2 : E-C3 hybrid thioredoxin.

tains pure E-C3 hybrid thioredoxin. The purified E-C3 hybrid thioredoxin was found to have a molecular weight of 12,000 daltons, which corresponded to the size estimated from the nucleotide sequence. This preparation was used for the characterization of E-C3 hybrid thioredoxin.

The E-C3 hybrid thioredoxin contains the N-terminal sequence of the *E. coli* thioredoxin and the C-terminal sequence of *C. nephridii* thioredoxin C-3. Estimated from their predicted amino acid sequences, the E-C3 hybrid thioredoxin contained two half cystines and consisted of 113 amino acid residues. On native 12% polyacrylamide gels, the purified E-C3 hybrid thioredoxin exhibits a much lower mobility than *E. coli* thioredoxin (Fig. 5). This delayed migration may be attributed to the C-terminal region of the E-C3 hybrid thioredoxin, which has many positive-charged amino acid residues.

E. coli thioredoxin is a substrate for *E. coli* thioredoxin reductase (Holmgren, 1985). Kinetic measurements of the reaction of the purified E-C3 hybrid thioredoxin with *E. coli* thioredoxin reductase were carried out by using a Model UV-240 Shimadzu spectrophotometer at 25℃. The kinetic parameters were obtained from Lineweaver-Burk plots. As shown in Table 2, the E-C3 hybrid thioredoxin exhibits a 40-fold lower catalytic efficiency with *E. coli* thioredoxin reductase than *E. coli* thioredoxin. The purified E-C3 hybrid thioredoxin has a 10-fold higher K_m value than *E. coli* thioredoxin, indicating poor interaction with *E. coli* thioredoxin reductase. Lim *et al.* (1988), using Anabaena 7119/*E. coli* hybrid thioredoxins, have demonstrated that the C-terminal portions of thioredoxin are very critical for the interaction with ribonucleotide reductase, thioredoxin reductase and T7 phage gene-5 protein. Comparing

Table 2. Reduction of Thioredoxins with *Escherichia coli* Thioredoxin Reductase[a]

Thioredoxins	K_m (μM)	V_{max} (μmol/min)	Catalytic efficiency (V_{max}/K_m)
E. coli thioredoxin	0.67	6.67×10^{-2}	9.96×10^{-2}
E-C3 hybrid thioredoxin	6.67	1.67×10^{-2}	0.25×10^{-2}

[a]Assay mixtures contained 100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 50 μg of bovine serum albumin, 0.5 mM DTNB, and 0.24 mM NADPH in a volume of 1.0 ml. *E. coli* thioredoxin was added in a concentration range from 0.13 to 0.50 μM and E-C3 hybrid thioredoxin was added in a concentration range from 0.83 to 3.33 μM. The reaction was initiated by adding *E. coli* thioredoxin reductase, the increase in A_{412} was monitored at 25° C, and activities were calculated as $OD_{412}\times min^{-1}$.

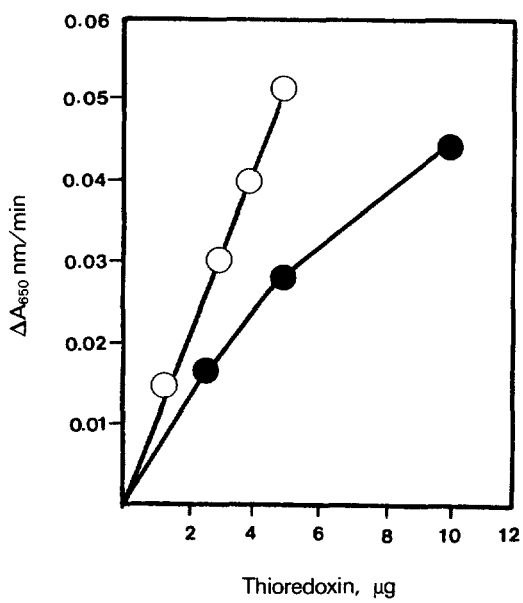

Fig. 6. Insulin reduction activity of purified E-C3 hybrid thioredoxin. Insulin reduction was assayed by turbidity formation at 25 ℃. The assay mixtures contained, in a final volume of 600 μl, 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, 0.13 mM bovine insulin, 0.33 mM dithiothretol, and *E. coli* thioredoxin (○–○) or E-C3 hybrid thioredoxin (●–●).

the catalytic efficiency of *E. coli* and E-C3 hybrid thioredoxins, the *E. coli* thioredoxin is a much superior substrate for *E. coli* thioredoxin reductase. This difference is mainly due to changes in the K_m's. The less efficient binding of the E-C3 hybrid thioredoxin to *E. coli* thioredoxin reductase has been attributed to altered residues in the C-terminal domain of the E-C3 hybrid thioredoxin. The kinetic data shows that the C-terminal portion of *E. coli* thioredoxin is critical for the interaction with *E. coli* thioredoxin reductase.

Thioredoxins can reduce insulin disulfides (Holmgren, 1979). To estimate the ability of the hybrid thioredoxin to function as a protein disulfide reductase, its insulin

precipitation activity was determined in the presence of dithiothreitol (Method 2 under Materials and Methods). The results shown in Fig. 6 indicate that the purified E-C3 hybrid thioredoxin is able to reduce insulin. Turbidity formation was increased in a thioredoxin concentration-dependent manner (Fig. 6). Insulin precipitation activity of the E-C3 hybrid thioredoxin was low compared to wild-type *E. coli* thioredoxin. This low activity may be due to the interfering effect of the C-terminal region of the E-C3 hybrid thioredoxin. This data shows that the purified E-C3 hybrid thioredoxin functions as a protein disulfide reductase.

In conclusion, we purified and characterized the E-C3 hybrid thioredoxin from the *Escherichia coli* thioredoxin-deficient strain harboring a plasmid encoding the E-C3 hybrid thioredoxin. Purified E-C3 hybrid thioredoxin was homogeneous with a molecular weight of 12,000 as determined by SDS polyacrylamide gel electrophoresis. On native polyacrylamide gels, the purified E-C3 hybrid thioredoxin exhibits a much lower mobility than *E. coli* thioredoxin. Purified E-C3 hybrid thioredoxin was able to reduce insulin. The E-C3 hybrid thioredoxin shows a 40-fold lower catalytic efficiency than *E. coli* thioredoxin. Our results may indicate that interaction of two domains in thioredoxins is important in their functioning. Some activities of thioredoxin may require a precise 3-dimensional structure of the protein. Further study should be done to elucidate the structure-function relationship of thioredoxins.

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