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Direct Reduction of DTNB by *E. coli* Thioredoxin Reductase

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Abstract: Thioredoxin reductase is a flavoprotein oxidoreductase catalyzing the reduction of a cystine disulfide in thioredoxin. Thioredoxin, in turn, can reduce disulfide bonds in other proteins and serves as a reducing agent in enzymatic reactions such as those of ribonucleotide reductase and methionine sulfoxide reductase. In this work thioredoxin reductase was found to directly reduce DTNB in the absence of thioredoxin. This new reactivity of *E. coli* thioredoxin reductase was produced by relatively high concentrations of univalent cations such as Na^+, K^+, Li^+, and NH_4^+, and it appeared with the oxidation of NADPH. These results indicate that *E. coli* thioredoxin reductase may be slightly modified by univalent cations, and the modified enzyme directly reacts with DTNB. This DTNB-reducing activity offers a new assay method for *E. coli* thioredoxin reductase.

Key words: Thioredoxin reductase, *Escherichia coli*, thioredoxin, DTNR.

Thioredoxin reductase is an FAD-containing enzyme which belongs to a family of FAD-disulfide oxidoreductases, including glutathione reductase, dihydrolipoamide dehydrogenase, trypanothione reductase, and mercuric ion reductase(Holmgren, 1985). It catalyzes the transfer of electrons between NADPH and the disulfide of thioredoxin (Moore *et al.*, 1964; Zanetti and Williams, 1967). Since thioredoxin reductase was isolated originally from *Escherichia coli* (Moore *et al.*, 1964), it has been purified to homogeneity from Novikoff ascites rat tumor (Chen *et al.*, 1977), rat liver (Luthman and Holmgren, 1982), calf liver (Martînez-Galisteo *et al.*, 1992), and calf thymus (Kumar *et al.*, 1992). *E. coli* thioredoxin reductase has an M_r value of 70,000 and consists of two identical subunits linked by noncovalent bonds (Ronchi and Williams, 1972). The subunits each contain an active center disulfide sequence of -Cys-Ala-Thr-Cys- (Thelander, 1970). The *E. coli trxB* gene encoding thioredoxin reductase was isolated and its nucleotide sequence was determined (Russel and Model, 1985; Russel and Model, 1988). In the predicted amino acid sequence the three short segments, two of which are probably involved in FAD and NADPH binding, are highly homologous with the sequences of glutathione reductase (Greer and Perham, 1986) and dihydrolipoamide reductase (Stephens *et al.*, 1983).

The catalytic sequence of thioredoxin reductase is divided into two half-reactions. The first half-reaction is a nucleophilic attack by one enzyme thiol on the disulfide of thioredoxin to form a mixed disulfide bridge between the enzyme and substrate. The second step involves a nucleophilic attack by the other enzyme active site thiol on the mixed disulfide to generate a disulfide on thioredoxin reductase, and release of reduced thioredoxin. Rapid reduction of the disulfide bond in thioredoxin by thioredoxin reductase proceeds at pH 7.0 (Moore *et al.*, 1964). The activities of calf-liver and *E. coli* thioredoxin reductases are drastically decreased in the presence of NADPH or NADH, while $NADP^+$, NAD^+ and oxidized *E. coli* thioredoxin significantly activate both enzymes (Martînez-Galisteo *et al.*, 1992). Selenite (Kumar *et al.*, 1992) and protein disulfide-isomerase (Lundstrôm and Holmgren, 1990) serve as substrates for thioredoxin reductase. This paper describes *E. coli* thioredoxin reductase reduction of DTNB in the presence of univalent cations and NADPH. These results indicate *E. coli* thioredoxin reductase has a DTNB-reducing activity in a certain environment.

Materials and Methods

Materials

5,5-Dithio-bis(2-nitrobenzoic acid)[DTNB], bovine serum albumin (BSA), NADPH, tris(hydroxymethyl) aminomethane, and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Co. *E. coli* thioredoxin was purified as described by Lim *et*

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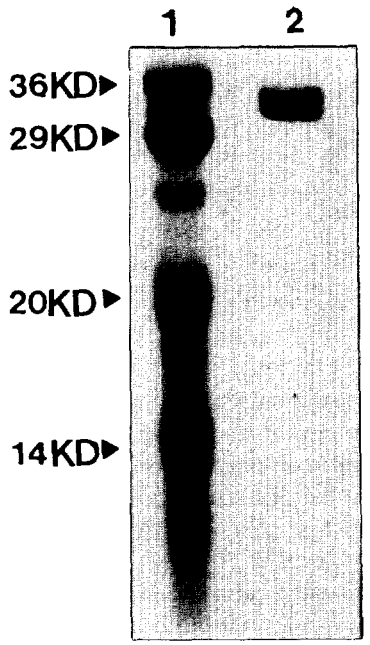

Fig. 1. 12% SDS-polyacrylamide gel electrophoretic pattern of purified *E. coli* thioredoxin reductase used in the present study. Lane 1 : Marker Proteins; Lane 2 : Purified *E. coli* thioredoxin reductase.

al. (1985) from an overproducing strain containing the *trxA* gene in a pUC vector. *E. coli* thioredoxin reductase was obtained from an overproducing strain harboring the *trxB* gene encoding thioredoxin reductase in a pUC vector (Russel and Model, 1985). The SDS-polyacrylamide gel electrophoretic pattern of *E. coli* thioredoxin reductase used in this study is shown in Fig. 1. All other reagents were of analytical grade, or the highest quality commercially available.

Assay of *E. coli* thioredoxin reductase

DTNB assay : The native activity of thioredoxin reductase was assayed at 25℃ by a DTNB-coupled assay, which measures FAD-mediated electron transfer between NADPH and *E. coli* thioredoxin (Moore *et al.*, 1964). The reaction mixture contained 0.1 M Tris-Cl (pH 8.0), 0.5 mM DTNB, 0.24 mM NADPH, 0.1 mg/ml BSA, 2 mM EDTA, *E. coli* thioredoxin and thioredoxin reductase. The reaction was started by adding thioredoxin reductase, and the absorbance increase at 412 nm was recorded.

Direct reduction of DTNB : The DTNB-reducing activity of thioredoxin reductase was assayed in the presence of DTNB as a substrate. The reaction mixture contained 0.1 M Tris-Cl (pH 8.0), 0.5 mM DTNB, 0.24 mM NADPH, 0.1 mg/ml BSA, and thioredoxin reductase. The reaction was started by adding thioredoxin reductase, and the absorbance change at 412 nm was directly monitored. Absorbance measurements were made with a UV-VIS Shimadzu Spectrometer (Model UV-240).

Table 1. Effect of various salts on the direct DTNB reduction by *E. coli* thioredoxin reductase[a]

Salts	ΔOD_{412}/min
–	0.000
1 M NaCl	0.018
1 M KCl	0.018
1 M $(NH_4)_2SO_4$	0.014
0.5 M Li_2SO_4	0.020
1 M $CaCl_2$	0.000
1 M $MgCl_2$	0.000
0.5 M $AlCl_3$	0.000

[a]Salts were added to the reaction mixture (1 ml) containing 0.1 M Tris-Cl (pH 8.0), 0.5 mM DTNB, 0.24 mM NADPH, and 0.1 mg BSA. The reaction was initiated by adding 0.2 μg *E. coli* thioredoxin reductase. The increase at OD_{412} was directly monitored at 25℃.

Results and Discussion

This paper describes DTNB as a substrate for *E. coli* thioredoxin reductase in the presence of univalent cations. DTNB is primarily known to serve as a substrate for thioredoxins (Luthman and Holmgren, 1982), and is used together with thioredoxin for detecting thioredoxin reductase activity. Thioredoxin reductase can also be assayed in the presence of thioredoxin and insulin (Luthman and Holmgren, 1982). This type of assay is based on the protein disulfide reductase activity of thioredoxin. The two coupled assay methods for thioredoxin reductase require another protein, thioredoxin.

E. coli thioredoxin reductase directly reduced DTNB in the presence of the univalent cations Na^+, K^+, NH_4^+, and Li^+ (Table 1). However, the divalent cations Ca^{2+} and Mg^{2+} and the trivalent cation (Al^{3+}) did not cause thioredoxin reductase reduction of DTNB. The direct reduction of DTNB by thioredoxin reductase did not occur without NADPH. In an original assay system, *E. coli* thioredoxin and thioredoxin reductase greatly decreased DTNB reduction in the presence of a high concentration of NaCl (data not shown). In the reduction of DTNB by *E. coli* thioredoxin reductase, NADPH oxidation did not take place in the absence of a high concentration of NaCl(Table 2). This indicates that DTNB reduction by *E. coli* thioredoxin reductase requires NADPH as a final electron acceptor.

DTNB reduction by *E. coli* thioredoxin reductase appears to occur in a cation concentration-dependent manner. Fig. 2 shows that DTNB reduction by *E. coli* thioredoxin reductase reaches a saturation level at 0.5 M NaCl. The DTNB reducing activity of *E. coli* thiore-

Table 2. NADPH oxidation by *E. coli* thioredoxin reductase in the presence of DTNB and NaCl

Substance added	NADPH oxidation
Standard[a]	+
−NaCl	−
−Thioredoxin reductase	−

[a] The standard reaction mixture contains 0.1 M Tris-Cl (pH 8.0), 0.5 M NaCl, 0.5 mM DTNB, 0.24 mM NADPH, 0.1 mg BSA, and 0.2 μg *E. coli* thioredoxin reductase. The absorbance change at 340 nm was directly monitored at 25℃.

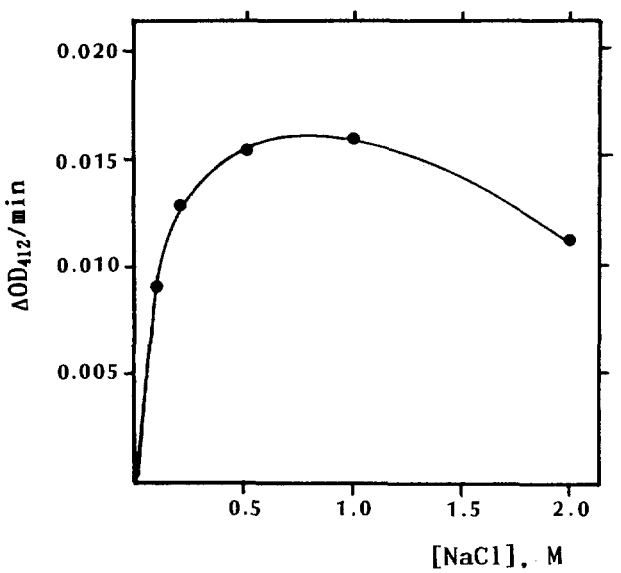

Fig. 2. Concentration effect of NaCl on the direct reduction of DTNB by *E. coli* thioredoxin reductase. Assays were done at pH 8.0 as described under "Materials and Methods". The amount of *E. coli* thioredoxin reductase used was 0.2 μg.

doxin reductase is proportional to the amount of the enzyme (Fig. 3). This linear relationship indicates that the DTNB reducing activity is essentially the result of thioredoxin reductase. Additionally it suggests that DTNB reducing activity can be used for assaying *E. coli* thioredoxin reductase.

A Lineweaver-Burk plot for *E. coli* thioredoxin reductase with DTNB as a substrate is shown in Fig. 4. From the plot the K_m value is 5 mM and the V_{max} value is 0.13 (ΔOD_{412}/min). The K_m value obtained is relatively high, indicating that *E. coli* thioredoxin reductase has a low affinity for DTNB. The temperature effect was also examined in the direct reduction of DTNB by *E. coli* thioredoxin reductase in the presence of 0.5 M NaCl (Fig. 5). DTNB reduction is highest at 10℃, among the temperature points tested. This temperature-dependent character indirectly confirms the enzymatic DTNB reduction by *E. coli* thioredoxin reductase.

In this work *E. coli* thioredoxin reductase was found

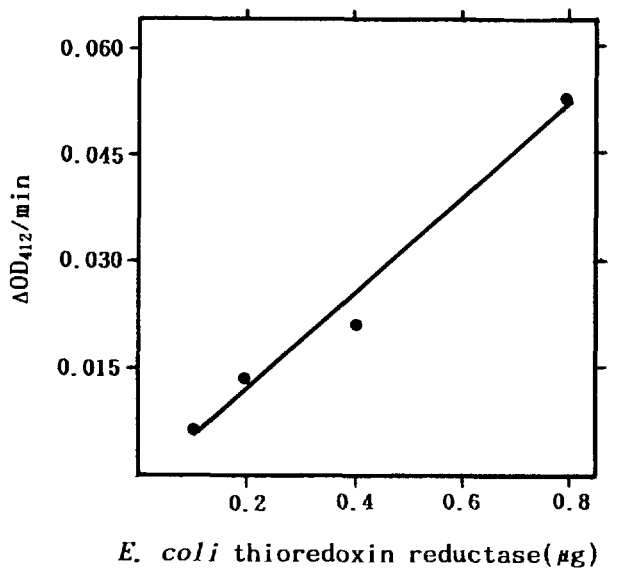

Fig. 3. The linear relationship between DTNB reduction and *E. coli* thioredoxin reductase content in the presence of 0.5 M NaCl. Assays were done in various contents of *E. coli* thioredoxin reductase at pH 8.0 as described under "Materials and Methods".

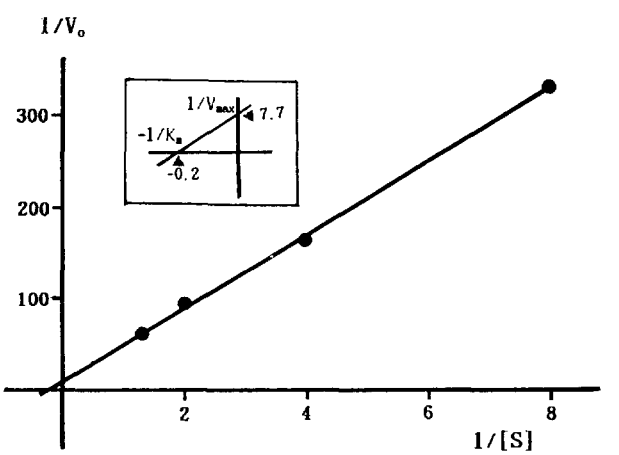

Fig. 4. Lineweaver-Burk plot of DTNB as a substrate for *E. coli* thioredoxin reductase. Assays were done at pH 8.0 as described under "Materials and Methods". The amount of *E. coli* thioredoxin reductase used was 0.2 μg. The concentrations of DTNB were 0.125 mM, 0.25 mM, 0.5 mM, and 0.75 mM. The intersection region was enlarged in the inset.

to directly reduce DTNB in the presence of univalent cations. However, *E. coli* thioredoxin reductase does not catalyze the reduction of insulin disulfide by dithiothreitol, even together with univalent cations (data not shown). As illustrated in Fig. 3, the reduction of DTNB by *E. coli* thioredoxin reductase can be an easy assay method for *E. coli* thioredoxin which does not require thioredoxin. Univalent cations are required for the DTNB reducing activity of *E. coli* thioredoxin reductase, whereas divalent and trivalent cations are not needed for its DTNB reducing activity. This fact suggests that

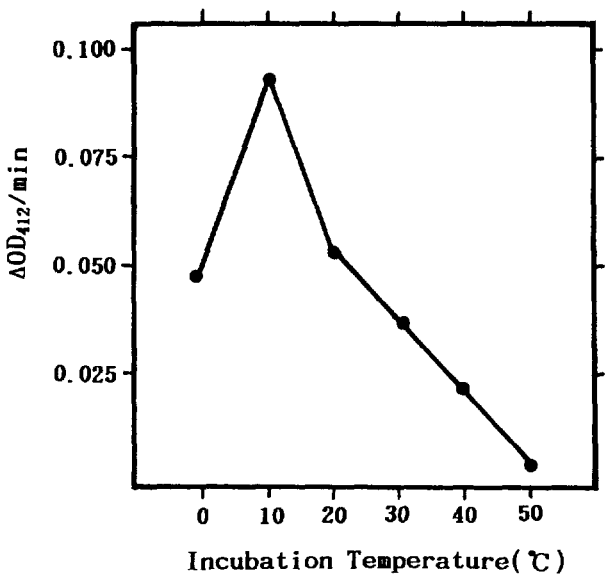

Fig. 5. The temperature effect on the direct reduction of DTNB by *E. coli* thioredoxin reductase in the presence of 0.5 M NaCl. The assay mixture contains 0.1 M Tris-Cl (pH 8.0), 0.5 mM DTNB, 0.24 mM NADPH, 0.1 mg BSA, and 0.4 μg *E. coli* thioredoxin reductase. The mixtures were incubated at various temperatures for 5 min. After incubation, the absorbance was measured at 412 nm.

ionic strength does not play an important role in DTNB reducing activity. Univalent cations may induce a conformational change in *E. coli* thioredoxin reductase, in which its closed conformation is converted to an open conformation. If this is true the active site would be exposed in an open conformation of *E. coli* thioredoxin reductase. Based on this mechanism the exposed active site of thioredoxin reductase may make a direct reaction with DTNB possible. Further study should be done to elucidate why *E. coli* thioredoxin reductase uses DTNB as a substrate in the presence of univalent cations.

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