Cloning, Expression, and Characterization of a Novel *Escherichia coli* Thioredoxin* 

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Thioredoxin (Trx) is a small ubiquitous protein that displays different functions mainly via redox-mediated processes. We here report the cloning of a gene (*trx*) coding for a novel thioredoxin in *Escherichia coli* as well as the expression and characterization of its product. The gene encodes a protein of 139 amino acids (*Trx*2) with a calculated molecular mass of 15.5 kDa. *Trx*2 contains two distinct domains: an N-terminal domain of 32 amino acids including two CXXC motifs and a C-terminal domain, with the conserved active site, Trp-Cys-Gly-Pro-Cys, showing high homology to the prokaryotic thioredoxins. *Trx*2 together with thioredoxin reductase and NADPH is an efficient electron donor for the essential enzyme ribonucleotide reductase and is also able to reduce the interchain disulfide bridges of insulin. The apparent *Kₐ* value of *Trx*2 for thioredoxin reductase is similar to that of the previously characterized *E. coli* thioredoxin (Trx1). The enzymatic activity of *Trx*2 as a protein-disulfide reductase is increased by preincubation with dithiothreitol, suggesting that oxidation of cysteine residues other than the ones in the active site might regulate its activity. A truncated form of the protein, lacking the N-terminal domain, is insensitive to the presence of dithiothreitol, further confirming the involvement of the additional cysteine residues in modulating *Trx*2 activity. In addition, the presence of the N-terminal domain appears to confer heat sensitivity to *Trx*2, unlike Trx1. Finally, *Trx*2 is present normally in growing *E. coli* cells as shown by Western blot analysis.

Thioredoxin (*Trx*) is a small protein (Mᵣ 12,000) with a conserved active site sequence Trp-Cys-Gly-Pro-Cys that catalyzes many redox reactions through the reversible oxidation of its active site dithiol to a disulfide. Oxidized thioredoxin, *Trx*-S₂, can be reduced by NADPH and the flavoenzyme thioredoxin reductase, the so-called thioredoxin system (Reaction 1) (1). Reduced thioredoxin, *Trx*-(SH)₂, contains two thiol groups and can efficiently catalyze the reduction of many exposed disulfides, thus being a general protein-disulfide reductase, Reaction 2.

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\begin{align*}
\text{REACTION 1} & \\
\text{REACTION 2} & \\
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Thioredoxin is present in all living organisms and has been isolated and characterized from a wide variety of prokaryotic and eukaryotic cells (1). In *Escherichia coli*, thioredoxin was first identified as an electron donor for ribonucleotide reductase (RNR), the enzyme that reduces ribonucleotides to deoxyribonucleotides for DNA synthesis and repair (2). *E. coli* thioredoxin can also function as a hydrogen donor for 3'-phosphoadenosine 5'-phosphosulfate reductase in the sulfate assimilation pathway as well as methionine sulfoxide reductase (3, 4). Apart from these functions, *E. coli* thioredoxin is necessary for the life cycle of some bacteriophages such as T7, M13, and FR1 (5–7). In eukaryotic cells, thioredoxin can also function as a hydrogen donor for RNR, 3'-phosphoadenosine 5'-phosphosulfate, and methionine sulfoxide reductases, similar to the prokaryotic thioredoxin (1). In addition, thioredoxin can (a) facilitate refolding of disulfide-containing proteins (8); (b) activate the interleukin-2 receptor (9); (c) modulate the DNA binding activity of some transcription factors, e.g. NF-xB (10); and (d) stimulate proliferation of lymphoid cells and a variety of human solid tumors (11, 12). Furthermore, thioredoxin is an efficient antioxidant able to reduce hydrogen peroxide (13), scavenge free radicals (14), and protect cells against oxidative stress (15). In photosynthetic organisms, three types of thioredoxins have been identified, two forms in chloroplasts (f and m) involved in regulatory systems in oxygen photosynthesis and one form in cytosol and endoplasmic reticulum (b) (16).

*E. coli* Trx is a well studied enzyme, and its three-dimensional structure has been determined by NMR for both oxidized and reduced forms, as well as by x-ray crystallography (17, 18). *E. coli* thioredoxin contains 109 amino acids and has a central core of five strands of twisted β-pleated sheet flanked by four α-helices and the active site located in a protrusion of the protein (19).

Thioredoxin-negative mutants (*trx*Δ) of *E. coli* are viable (5), and analysis of these mutants led to the identification of a novel cofactor, glutaredoxin-1 (Grx1), as an efficient substitute of thioredoxin for RNR and 3'-phosphoadenosine 5'-phosphosulfate reductase enzymatic activity (20, 21). However, Grx1 could neither substitute for thioredoxin in methionine sulfoxide reduction nor in bacteriophage growth or assembly (5–7, 22), which thus remained typical phenotypes of *E. coli* thioredoxin mutants. The isolation of an *E. coli* double mutant in thio-
doxin/glutaredoxin-1 allowed the identification of two novel glutaredoxinas, Grx2 and Grx3, but only Grx3 is able to serve as a hydrogen donor for RNR (23, 24). Thus, it seemed that only one thioredoxin did exist in E. coli. However, the existence of another thioredoxin has been suggested to be necessary for the maintenance of the reducing environment in E. coli cytoplasm (25). In addition, a triple Trx, Grx1, and Grx3 mutant was viable (26), indicating the presence of an alternative protein capable of reducing RNR in vivo.

We report here the cloning of a DNA sequence coding for a novel E. coli thioredoxin (Trx2) based upon biological activity data and protein homology. We also present evidence that the protein is normally expressed in E. coli cells and that the N-terminal sequence of the protein contains a novel domain with four cysteine residues that partly regulates its enzymatic activity as protein-disulfide reductase.

MATERIALS AND METHODS

Strains and Media
E. coli K-12 was a stock from our laboratory. K38 (wild type) and A179 (trxA::kan) (27) strains were a kind gift from Prof. Arne Holmgren (Karolinska Institutet, Stockholm). Cells were grown in LB medium supplemented (when necessary) with 50 µg/ml ampicillin or kanamycin.

Cloning of the E. coli Thioredoxin 2
A thioredoxin-like sequence (Trx2) opening an reading frame coding for a protein of 139 amino acids (GenBankTM accession number 1788936), was used to design the specific mutagenic primers EcTrx2-f and EcTrx2-r. The chosen primer is normally expressed in E. coli Trx2 and can be used to amplify the specific Trx2 sequence. The PCR product was cloned into the pGEM-T Easy Vector System I (Promega) and sequenced.

Preparation of Cellular Fractions from E. coli

Laemmli Treatment—Cells were harvested in the previous treatment. The pellet was resuspended in the same buffer plus 2 mM MgCl2 and 2 mM lysozyme at an A590=40, incubated for 30 min on ice, and centrifuged at 14,000 rpm at 4 °C for 1 min. The supernatant was cleared by centrifugation, passed through a 0.22 µm filter, and sonicated for 30 s. The supernatant was assayed for thioredoxin reductase activity of thioredoxin as described previously (29).

Enzyme Activity Assays

Thioredoxin reductase activity was measured using the DTNB assay as described by Song et al. (28). Female chickens were injected (once every 15 days) with 200 ml of lysozyme specific anti-TNBS antibody serum albumin (BSA), 150 mM NaCl, and 0.1% Tween 20 and further incubated with affinity-purified anti-Trx2 antibodies. Immunodetection was performed with horse-radish peroxidase-conjugated rabbit anti-chicken IgG (Sigma) diluted 1:5000, following the ECL protocol (Amersham).

Enzyme Assays of E. coli Thioredoxin 2
The activity of E. coli Trx2 was determined by the DTNB and insulin assays. The DTNB assay was essentially as described elsewhere (29). Briefly, the reaction mix contained 200 mM phosphate buffer, pH 7.0, 2 mM EDTA, 0.1 mg/ml BSA, 1 mM DTNB, and 0.5 mM NADPH. The reactions containing 0.5–8 µM Trx1, Trx2, or ΔTrx2 were started by the addition of 10 nM E. coli thioredoxin reductase (IMCO, Sweden). The reaction was followed at 412 nm against a blank containing thioredoxin reductase in a SpectraMaxTM 190 Microplate Spectrophotometer (Molecular Devices Corp.) for 7 s, with an average final volume of 100 µl. Insulin was used to determine the protein-disulfide reductase activity of thioredoxin as described previously (29).

The rate of DTNB reduction was calculated from the increase in A412 using a molar extinction coefficient of 14,180 M−1 cm−1, since reduction of DTNB by 1 mol of Trx(ΔTrx2) yields 2 mol of 3-carboxy-4-nitrobenzoic acid with a molar extinction coefficient of 13,310 M−1 cm−1 (29).
A Novel E. coli Thioredoxin

Values of ΔA412 were multiplied by a factor of 4.3 to give the ΔA412 of a cuvette with a path length of 1 cm. Reduction by DTT was carried out by preincubation of aliquots of Trx2 at 37 °C for 20 min with 2 μl of 50 mM HEPES, pH 7.6, 100 μM/μl BSA, and 2 mM DTT.

Ribonucleotide Reductase Assay

The main difference in Trx2 protein sequence with respect to the well known Trx1 is the presence of an extra N-terminal domain of 32 amino acids at the N terminus. The C-terminal half of the protein contains the active site found in all thioredoxins (Fig. 2). Recently, Lim et al. (34) have described a third thioredoxin in Corynebacterium nephridii, which displays a similar structure to E. coli Trx2 with an extra N-terminal domain of 32 amino acids and a C-terminal domain homologous to the rest of the well known Trx1. The Trx2 protein contains the active site found in all thioredoxins (Fig. 2).

Enzymatic Activities of E. coli Thioredoxin 2—To establish that the putative Trx2 does in fact encode a protein with thioredoxin activity, we cloned the Trx2 gene into the pET-15b expression vector under the control of a T7 promoter. The resulting plasmid pET-Trx2 was transformed in E. coli BL21 (DE3), and the expression of His-Trx2 was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside for 3.5 h.
The recombinant protein was expressed to levels of approximately 20% of the total soluble protein and was purified almost to homogeneity by affinity chromatography with a Talon column (Fig. 3, inset). Recombinant Trx2 (with or without the His tag) was used to examine the reduction of insulin, a classical assay in which thioredoxin catalyzes disulfide reduction of insulin in a coupled reaction with NADPH in the presence of E. coli thioredoxin reductase.

As shown in Fig. 3, oxidized Trx2 was active as a disulfide reductase; however, it exhibited approximately 5-fold lower activity than Trx1 at concentrations between 0.2 and 0.5 mM and 1.5–2-fold lower activity between 0.5 and 2 mM. At these higher thioredoxin concentrations, the efficiency of thioredoxin reductase is the rate-limiting step, since the reactivity of Trx-(SH)2 and insulin is known to be very fast (35, 36).

To understand the role of the extra cysteines present at the N terminus of Trx2, we preincubated Trx2 with DTT. As shown in Fig. 4, the ability of Trx2 to reduce insulin was increased to levels similar to that of Trx1 (compare with Fig. 3). The fact that Trx2 activity was enhanced after reduction of the protein suggested that cysteine residues in Trx2 other than those in the active site could be involved in regulating its enzymatic activity. We used the truncated form of Trx2 (∆Trx2) lacking the N-terminal portion, including the four cysteine residues, to further test this possibility. Fig. 4 shows that ∆Trx2 has thioredoxin activity independent of DTT reduction, similar to Trx1. Furthermore, the activity is similar to the reduced Trx2 and higher than the one displayed by the full-length oxidized protein, indicating that the N-terminal portion of the protein partly regulates the activity of Trx2.

DTNB is an artificial disulfide substrate and a fast oxidant of Trx-(SH)2, which keeps the concentration of Trx-S2 constant. DTNB is often used in the assay of thioredoxin reductase, where one molecule of DTNB is split into two 5'-thionitrobenzoic acid molecules by reduced Trx1 (1). The DTNB assay and low concentrations (10 nM) of thioredoxin reductase to obtain saturation Michaelis-Menten kinetics were used to calculate the $K_m$ of Trx2 for E. coli thioredoxin reductase at pH 7.0 and 25 °C. The values obtained were used to calculate the $k_{cat}$ as well as the $k_{cat}/K_m$ or apparent second order rate constant for the reaction between thioredoxin reductase and Trx-S2. The $K_m$ value for Trx2 and the $k_{cat}/K_m$ were similar to Trx1. The ∆Trx2 has a lower $K_m$ value, and the $k_{cat}$ is approximately halved. Table I shows the comparison of Trx1 and Trx2 kinetic parameters.

Trx2 as an Electron Donor for Ribonucleotide Reductase—E. coli contains genetic information for three different ribonucleotide reductases. The NrdAB is active during aerobicosis. NrdDG is active during anaerobiosis and uses formate as an electron donor. NrdEF is a cryptic enzyme that uses Grx1 but not Trx1 as an electron donor (37–39). The viability of a triple mutant Trx1, Grx1 and Grx3, the three known electron donors for the NrdAB protein when growing in aerobicosis, prompted us to assay Trx2 as a tentative reductant for this enzyme. As shown in Fig. 5, Trx2 was found to be a functional electron donor for the NrdAB enzyme. However, compared with Trx1, Trx2 was almost 2.5-fold less efficient as an electron donor than Trx1. Similar to Trx1, Trx2 was not an electron donor for NrdEF (40).

Expression of E. coli Thioredoxin 2—To assay for the presence of Trx2 activity in E. coli, we used protein extracts from K38 (wild type) and A179 (trxA::kan) strains (27). To decrease the background in the insulin assay due to interaction of DTNB with -SH groups, we used heated extracts (85 °C, 5 min). Thioredoxin activity was detected in E. coli A179 (trxA·), although about 15-fold lower than the one displayed by the wild type (data not shown).

Affinity-purified polyclonal antibodies were used to further analyze the presence of Trx2 in E. coli extracts. Fig. 6A shows that the antibodies reacted with one band at the expected position of Trx2 in a total crude extract of E. coli (lane 3), indicating that the protein is normally expressed. These antibodies also reacted with E. coli Trx1, being detectable in the wild type strain but not in the mutant (Fig. 6A, compare lane 3).
with lane 4). However, antibodies raised against E. coli Trx1 did not cross-react with recombinant Trx2 (data not shown). The affinity of the anti-Trx2 antibodies for Trx1 was 10-fold lower than for Trx2 (Fig. 6B). From the immunoblot and densitometric analysis, the contents of Trx2 and Trx1 in the total crude extract of E. coli were estimated to be about 0.33 and 1.7 μg/mg protein, respectively. The calculated value for Trx1 is in good agreement with the amount of Trx1 (1.91 μg/mg protein) obtained by enzyme-linked immunosorbent assay (41).

Thus, the levels of Trx2 versus Trx1 were different when calculated from the Western blots (5-fold lower) than from the activity assays (15-fold lower). One possible explanation is the inactivation of Trx2 during the preparation of the heated bacteria extracts. Trx2 is a heat-stable protein, and heat extracts at 85 °C have been used previously to measure thioredoxin activity. We compared the heat stability of Trx2 versus Trx1 (Fig. 7). As expected, the activity of Trx1 was not affected after 5 min of treatment at increasing temperatures from 37 to 85 °C. In contrast, the activity of Trx2 was more dependent on temperature treatment, and after 5 min at 85 °C 40% of the insulin-reducing activity was lost. When the N-terminal part of Trx2 was deleted, the heat stability of Trx2 was similar to Trx1, indicating that the N-terminal part is responsible for the partial heat inactivation. Treatment with DTT after the heat treatment seems to restore protein activity.

**Subcellular Localization of E. coli Thioredoxin 2**—The N-terminal part of Trx2 does not match any consensus sequence for protein translocation to the periplasmic space. To determine whether Trx2 is a cytosolic or periplasmic protein, we used two different treatments, lysozyme and freeze-thaw. The lysozyme treatment, a test for defining strict periplasmic localization, disrupts the cell’s outer envelope, thus exposing the periplasmic space to the external environment. Freeze-thawing selectively releases cytosolic proteins attached to the inner surface of the cytoplasmic membrane into the periplasmic fraction (42). Cytosolic and periplasmic fractions obtained with lysozyme treatment (lanes 5 and 6, respectively) and cytosolic and periplasmic fractions obtained with lysozyme treatment (lanes

**Figure 4**. Effect of DTT on Trx2 insulin-reducing activity. DTT preincubation of different amounts of Trx2 and ΔTrx2 was carried out by adding 2 μl of DTT buffer (50 mM HEPES, pH 7.6, 100 μg/ml BSA, and 2 mM DTT) in a final volume of 20 μl and incubating for 20 min at 37 °C. Then reaction mix and E. coli thioredoxin reductase were added to a final volume of 60 μl to initiate the reaction. The reaction was stopped after 20 min by the addition of 6 M guanidine HCl, 1 mM DTNB.

**Figure 5**. Activity of Trx2 as electron donor for ribonucleotide reductases. The assay was performed with [3H]CDP as substrate in the presence of NADPH, thioredoxin reductase, and increasing concentrations of Trx1 and Trx2 as described under “Materials and Methods.” ○, Trx1 activity with NrdAB; ▲, Trx2 activity with NrdAB; △, Trx2 activity with NrdEF. Values are the average of two measurements in two independent experiments.

**DISCUSSION**

The identification of a novel thioredoxin sequence in the E. coli genome addresses the question of whether the protein coded by this sequence is active as thioredoxin and if it is normally expressed. We have tried to answer these questions and have demonstrated that this newly identified sequence codes for a protein with thioredoxin activity in the insulin, DTNB, and RNR assay. Also, this protein is expressed normally in E. coli cells as shown by Western blot analysis and activity assays.

The existence of a second thioredoxin-like protein in E. coli has been proposed by Beckwith and co-workers (25, 26). Thioredoxin reductase, the flavoenzyme that reduces thioredoxin via NADPH, is implicated in the maintenance of the reducing environment of the E. coli cytoplasm, since all mutants selected to allow disulfide bond formation in the cytoplasm mapped in the trxB gene (25), which codes for thioredoxin reductase. Nevertheless, E. coli thioredoxin reductase is a highly specific enzyme only capable of reducing thioredoxin and not any other disulfide bonds in cytoplasmic proteins. However, thioredoxin was not required for the maintenance of the reducing environment, since the single mutant trxA− showed no difference when compared with the wild type (25). These results indicated that

**Table 1**

|                | Kₘ  | Kₜₐₜ | kₜₐₜ/Kₘ |
|----------------|-----|-------|---------|
| Trx1           | 1.9 ± 0.2 | 11.3 ± 0.6 | 6.3 × 10⁶ |
| Trx2           | 2.4 ± 0.4 | 12.8 ± 1.0 | 5.4 × 10⁶ |
| Δ Trx2         | 1.5 ± 0.1 | 6.5 ± 0.5 | 4.7 × 10⁶ |

The assay was carried out as described under “Materials and Methods.” Three separate measurements were made for each protein, and the mean value derived from Lineweaver-Burk plots of 1/[S] versus 1/V is shown.
measurements were made for each protein, and the average values are

temperatures and then analyzed with the insulin assay. Two separate

maintenance of the reducing environment. The novel

protein, reducible by thioredoxin reductase and responsible for the

function. Additional evidence suggesting the existence of

Trx2 described here is an optimal candidate that could fulfill

this function. Whether all of these proteins can act as electron donors for

RNR under physiological conditions or whether some of them

support this function only when others are absent is a matter

for further study based on the characterization of the respec-

tive mutants.

Several attempts in the past to identify any residual thiore-

doxin activity by biochemical methods in E. coli mutants lack-

ing Trx1 have failed (20, 26, 41, 44). By assaying heated ex-

tracts (85 °C, 5 min) of K38 (wild type) and A179 (trxA−) for

activity with the insulin assay, we were able to identify a thioredoxin activity in the A179 extract that was approximately

15-fold lower than the one displayed by the wild type. Recently, Jordan et al. (43, 45) reported on the existence of a

glutaredoxin-like protein with thioredoxin-like activity in E. coli (NrdH). NrdH can be reduced by thioredoxin reductase and is almost as effective as Trx1 in reducing disulfide bonds in insulin. In addition, NrdH is an efficient electron donor for NdrAB and NrdEF enzymes with higher specificity for the latter. However, nrdH is located in the same poorly transcribed operon as nrdEF. Thus, the expression of NrdH is likely to be low, as in the case for NrdEF coded by a cryptic gene, and the protein could not be detected in E. coli. The fact that the levels of the thioredoxin activity in the trxA− mutant are in good agreement with the levels from the Western blot analysis also argues that this activity is due to Trx2.

The main difference between this novel Trx2 and the rest of the prokaryotic thioredoxins is the existence of 32 extra amino
 acids residues in its N terminus. This region of the protein does not match with any consensus sequence for translocation in E. coli, and the cytoplasmic localization of Trx2 was confirmed by Western blot analysis of periplasmic and cytosolic fractions. The co-localization of both thioredoxins suggests the attachment of Trx2 to the inner surface of the cytoplasmic membrane as it has been described previously for Trx1 (42). The levels of Trx2 were similar in the wild type and the trxA− mutant strain, indicating that the disruption of Trx1 does not significantly stimulate expression of Trx2. Also, the gene for Trx2 is positioned at 58.5 min, far away from the trxA gene, which is located at 84 min in the E. coli chromosome.

Interestingly, the N-terminal domain of E. coli Trx2 is hom-

ologous to the N terminus part of C. nephridii Trx3 (34). C. nephridii Trx3 could complement some thioredoxin-deficient E. coli mutant phenotypes (namely growth in minimal medium with methionine sulfoxide as the only methionine source and support of growth of bacteriophages T7 and M13 but not f1 growth), when cloned in a high expression vector. Thioredoxin-

1-deficient mutants of E. coli have several phenotypes that

should be expected to be complemented by the novel Trx2. The

maintenance of these phenotypes in the trxA mutant indicates

that either the function of Trx2 is different from that of Trx1 or the molecule number per cell of Trx2 is not enough to substi-
tute Trx1. Expression of Trx2 from a high expression vector in a trxA− strain is necessary to answer this question. E. coli Trx2

covered disulfide-reducing protein able to reduce RNR has been

proposed to exist in E. coli. Again, this novel thioredoxin could

also fulfill this function. In fact, our results showed that Trx2 is

an efficient electron donor for the NrdAB but not for the NrdEF

protein.

Reduction of ribonucleotides to their corresponding deoxyri-

bonucleotides is an essential step in all living organisms in

which the building blocks for DNA synthesis are supplied.

RNR, the enzyme responsible for this reaction is thus tightly

regulated, and the electron supply for its catalytic activity

must be guaranteed. If we consider the recently described NrdH

protein (see below) as an efficient electron donor for RNR (43),

E. coli Trx2 is the fifth electron donor identified for RNR.

Whether all of these proteins can act as electron donors for

RNR under physiological conditions or whether some of them

support this function only when others are absent is a matter

for further study based on the characterization of the respec-
tive mutants.

E. coli cytoplasm might contain another thioredoxin-like pro-

tein, reducible by thioredoxin reductase and responsible for the

maintenance of the reducing environment. The novel E. coli

Trx2 described here is an optimal candidate that could fulfill

this function. Additional evidence suggesting the existence of

another thioredoxin comes from a recent report that describes

the viability of a triple mutant of all of the known electron
donors for the essential enzyme RNR (26). Thus, a yet undis-



Fig. 6. Immunoblotting analysis. Samples were separated by

SDS-polyacrylamide gel electrophoresis (15%), blotted onto a nitrocel-

lulose membrane, and probed with anti-Trx2 affinity-purified antibod-

ies. A, identification of Trx2 in cell extracts of E. coli. Lane 1, E. coli

Trx1 (50 ng); lane 2, E. coli Trx2 (5 ng), where the His tag was removed

by thrombin; lane 3, K38 (wild type) total crude extract (15 μg); lane 4,

A179 (trxA−) total crude extract (15 μg); lane 5, pellet after freeze-thaw

treatment (5 μg); lane 6, supernatant after freeze-thaw treatment (5 μg);

lane 7, pellet after lysozyme treatment (10 μg); lane 8, supernatant

after lysozyme treatment (7 μg). B, affinity of anti-Trx2 antibodies for

Trx2. Similar results were obtained from three independent

experiments.

Fig. 7. Rate of insulin reduction as a function of temperature.

0.5 μg of Trx1, Trx2, and ΔTrx2 were incubated for 5 min at different

temperatures and then analyzed with the insulin assay. Two separate

measurements were made for each protein, and the average values are

shown. ●, Trx2; ○, Trx2 plus DTT; ■, ΔTrx2; △, Trx1.
and C. nephriddi Trx3 are the only examples where prokaryotic thioredoxins have two extra pairs of CXXC motifs. We showed that the activity of Trx2 is dependent on the redox state of the protein, since preincubation with DTT increases insulin reduction. Moreover, elimination of the N-terminal region of the protein not only abolished the DTT dependence but also rendered the protein more resistant to heat inactivation. Site-directed mutagenesis of these cysteine residues is needed to elucidate their role in this redox regulation.

It is also interesting to point out that the disposition of the four cysteine residues in the N terminus of Trx2 resembles the structure of a zinc finger. Zinc finger motifs have been described in various DNA-binding proteins, and the importance of the cysteine residues is supported by the observation that modification of these residues inhibited enzymatic activity (46). E. coli primase is an example of a bacterial protein with only one zinc finger domain that binds specific sequences in the DNA (47). In fact, the zinc finger domain of primase is located in the N terminus of the protein, similar to E. coli Trx2. We are currently exploring the possibility that E. coli Trx2 might contain a zinc finger motif.

The C-terminal part of the E. coli Trx2 is highly homologous to the rest of the prokaryotic thioredoxins with many amino acid residues conserved apart from those of the active site, including those necessary for protein-protein interactions or for maintenance of the native structure of the molecule. There is a Pro-108 at the position equivalent to Pro-76 in Trx1 that may bend in proteins. Additionally, they are highly conserved in thioredoxins and fulfill this function (Gly-84, Gly-92, and Pro-64 in Trx1). There are also Gly residues in Trx2 (Gly-116, Gly-124); however, there is an Arg-96 at the corresponding position of Pro-64.

Trx2 is an atypical thioredoxin with an N-terminal extension whose potential functions still represent an open question. Although the biological function of Trx2 remains unknown, maintenance of the reducing environment in cytoplasm is likely to be such a function. Determination of the redox potential of Trx2 as well as disruption of its gene are necessary to answer these questions. Whether Trx2 is represented in higher organisms than prokaryotes will also be of great interest. We answer these questions. Whether Trx2 is represented in higher organisms than prokaryotes will also be of great interest. We answer these questions.