Articles

Characterization of *Escherichia coli–Anabaena* sp. Hybrid Thioredoxins†

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ABSTRACT: Thioredoxin is a small redox protein with an active-site disulfide/dithiol. The protein from *Escherichia coli* has been well characterized. The genes encoding thioredoxin in *E. coli* and in the filamentous cyanobacterium *Anabaena* PCC 7119 have been cloned and sequenced. *Anabaena* thioredoxin exhibits 50% amino acid identity with the *E. coli* protein and interacts with *E. coli* enzymes. The genes encoding *Anabaena* and *E. coli* thioredoxin were fused via a common restriction site in the nucleotide sequence coding for the active site of the proteins to generate hybrid genes, coding for two chimeric thioredoxins. These proteins are designated *Anabaena–E. coli* (A–E) thioredoxin for the construct with the *Anabaena* sequence from the N-terminus to the middle of the active site and the *E. coli* sequence to the C-terminus, and *E. coli–Anabaena* (E–A) for the opposite construct. The gene encoding the A–E thioredoxin complements all phenotypes of an *E. coli* thioredoxin-deficient strain, whereas the gene encoding E–A thioredoxin is only partially effective. Purified E–A thioredoxin exhibits a much lower catalytic efficiency with *E. coli* thioredoxin reductase and ribonucleotide reductase than either *E. coli* or *Anabaena* thioredoxin. In contrast, the A–E thioredoxin has a higher catalytic efficiency in these reactions than either parental protein. Reaction with antibodies to *E. coli* and *Anabaena* thioredoxins shows that the antigenic determinants for thioredoxin are located in the C-terminal part of the molecule and retain the native conformation in the hybrid proteins. Differences in activity of the hybrid thioredoxins are attributed to altered interactions of the N- and C-terminal domains of the molecule which produce changes in the three-dimensional structure of the active-site region.

Thioredoxin from *Escherichia coli* is a small (M_r ~12000) redox protein with the active-center amino acid sequence -Trp-Cys-Gly-Pro-Cys-. The dithiol form of thioredoxin can serve as a reducing agent for *E. coli* ribonucleotide reductase and methionine sulfoxide reductase. In addition, this protein can function as a general protein disulfide reductase. Oxidized *E. coli* thioredoxin is reduced by NADPH, catalyzed by the flavoprotein thioredoxin reductase [see Holmgren (1985) for a review]. The bacterial thioredoxin is also required for the replication or assembly of some coliphages. The T7 gene 5 protein forms a one-to-one complex with the host thioredoxin (Modrich & Richardson, 1975). The complex is a highly effective DNA polymerase (Tabor et al., 1986). *E. coli* thioredoxin also plays some unknown role in the assembly of filamentous viruses M13 and f1 (Russel & Model, 1985; Lim et al., 1985b). The gene encoding thioredoxin has been cloned and sequenced (Wallace & Kushner, 1984; Höög et al., 1984; Lim et al., 1985a; Tabor et al., 1986). Site-directed mutagenesis that eliminates the redox activity of thioredoxin has been reported. *E. coli* strains which produce these altered proteins can still support the replication of f1 and T7 bacteriophages (Huber et al., 1986). These results suggest that it is the overall conformation of reduced thioredoxin which is required for viral growth rather than the redox function per se.

Thioredoxin from the cyanobacterium *Anabaena* 7119 is quite similar to *E. coli* thioredoxin and exhibits 49% amino acid identity to this protein, as illustrated in Figure 1. Conserved sequences are clustered in the region of the active site and in the C-terminal portion of the molecule (Gleason et al., 1985). From preliminary model building, *Anabaena* thioredoxin is expected to have a similar three-dimensional structure to the *E. coli* protein which is illustrated in Figure 2 (Gleason, 1986). The gene encoding thioredoxin from *Anabaena* was cloned into *E. coli* by selection for complementation of an *E. coli* mutant deficient in thioredoxin (*trxA*). A *trxA* mutant forms significantly smaller colonies on enriched plates, cannot support the replication of M13, f1, and T7 phage, and cannot use methionine sulfoxide as a methionine source. A *trxA* strain containing a plasmid encoding the *Anabaena* thioredoxin was indistinguishable from $trxA^+$ strains for all phenotypes except for the growth of phage. M13 and f1 were able to replicate but formed smaller plaques while T7 failed to form plaques. The DNA sequence of the *Anabaena* gene encoding thioredoxin was 55% homologous to the *E. coli* thioredoxin gene (Lim et al., 1986).

Since *E. coli* and *Anabaena* thioredoxins appear to be very similar in both structure and function despite only a 50% homology, it was of interest to generate reciprocal hybrid thioredoxins. An *Ava*II restriction site located in the codons between the two active-site cysteines was used for gene fusions. The resulting genes produced hybrid thioredoxins (A–E) having the *Anabaena* N-terminal sequence to the active site (approximately one-third of the molecule) and the *E. coli* amino acid sequence from the active site to the C-terminus. The other hybrid (E–A) is the reciprocal construct. In a *trxA* strain, the gene encoding the A–E thioredoxin completely

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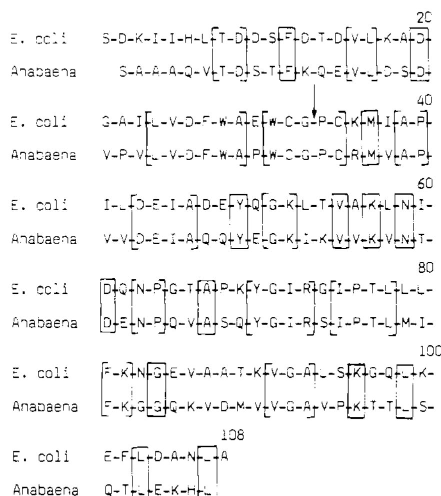


FIGURE 1: Amino acid sequence of *E. coli* and *Anabaena* thioredoxins. Alignment was made from the identical active-site disulfide ring, residues 31–35. Identical residues are enclosed in brackets. Numbering is from the *E. coli* sequence. The arrow marks the site at which the hybrid proteins were fused.

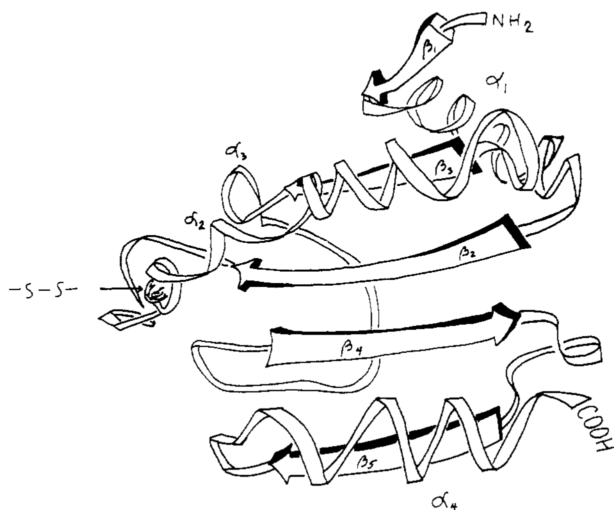


FIGURE 2: Schematic drawing of the three-dimensional structure of *E. coli* thioredoxin. Hybrid thioredoxins were constructed by cutting the genes at a site in the disulfide loop codons. The resulting hybrid proteins thus consist of β_1, α_1, and β_2 structures from *E. coli* and the remaining structural features from *Anabaena* thioredoxin in the E–A hybrid and the opposite construction for the A–E thioredoxin. The drawing is a simplified adaptation from the original which appeared in Holmgren et al. (1975).

restores the wild-type phenotype. However, in contrast to strains containing either the *E. coli* or the *Anabaena* thioredoxins, the strain containing the E–A hybrid was unable to support any viral growth except for an unusual mutant T7*tas* phage.

The hybrid thioredoxins were also purified and analyzed in vitro to determine the structural requirements and mechanism of action of thioredoxin. The A–E hybrid thioredoxin is a superior substrate for *E. coli* thioredoxin reductase and exhibits higher catalytic efficiency with *E. coli* ribonucleotide reductase than either parental protein. The opposite construct, E–A thioredoxin, is a relatively poor electron-transfer agent. The chemical synthesis of hybrid proteins to study structure–function relationships has been previously attempted. For example, the interaction of ovomucoid inhibitor (third domain) with serine proteases was probed with N–C-terminal hybrids of two natural variants (Wieczorek & Laskowski, 1983). A comparison of equilibrium constants of synthetic variants vs natural inhibitors enabled the authors to conclude that there are no long-range conformational effects on the efficiency of inhibitor binding. In contrast, our investigation of genetically produced hybrids illustrates a pronounced effect on catalytic efficiency, either positive or negative, due to subtle changes in domain interaction.

Materials and Methods

Materials. All restriction endonucleases, T4 DNA ligase, agarose, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal),[1] and isopropyl β-thiogalactoside (IPTG) were purchased from Bethesda Research Laboratories Inc., Gaithersburg, MD, or Boehringer Mannheim Biochemicals, Indianapolis, IN. All enzymes were used as specified by the manufacturer. Dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, bovine insulin, acrylamide, and anti-rabbit IgG alkaline phosphatase conjugate were from Sigma Chemical Co., St. Louis, MO. YM-5 ultrafiltration membranes (molecular weight cutoff 5000) were obtained from Amicon Corp., Lexington, MA. Bradford reagent and nitrocellulose paper were products of Bio-Rad Laboratories, Richmond, CA. [5-^{3}H]CDP was purchased from New England Nuclear, Boston, MA. Unlabeled nucleotides were obtained from P-L Laboratories, Milwaukee, WI. *E. coli* thioredoxin and *Anabaena* thioredoxin were purified from *E. coli* strains containing plasmid-encoded thioredoxins by previously published procedures (Holmgren & Reichard, 1967; Lim et al., 1986). *E. coli* thioredoxin reductase was purified on 2′,5′-ADP–Sepharose as described by Luthman and Holmgren (1982). A homogeneous preparation of *E. coli* ribonucleotide reductase was a generous gift of B. M. Sjöberg, Uppsala, Sweden.

Bacterial Strains and Phages. A thioredoxin-deficient (*trxA*$^-$) strain, BH2012, which is *E. coli* K-12,F$^-$,*ara*D139?,*gal*U,*gal*K,*hsr*,*rps*L, *met*A46,*arg*H1,*trx*A7004,*ilv*C::Tn5, was used as a recipient for transformation. As previously reported (Lim et al., 1985a), *trxA*$^+$ derivatives of BH2012 can be identified by increased colony size when plated on rich medium. BH2012 is unable to use methionine sulfoxide as a methionine source, whereas a *trxA*$^+$ derivative can. This phenotype was used to test the ability of a thioredoxin construct to serve as a cofactor for methionine sulfoxide reductase in *E. coli*. Derivatives of BH2012 were also used to test for the growth of bacteriophage T7. Strain BH5262, which is *E. coli* K-12,F$^-$,*ara*D139?,*gal*U,*gal*K,*hsr*,*rps*L,*arg*H1,*trx*A7004,*gsh*A,*srl*::*Tn*10, was also used for complementation tests. BH5262 is unable to grow on minimal medium unless supplemented with glutathione. Derivatives of BH5262 that are either *Gsh*$^+$ or *TrxA*$^+$ do not require glutathione for growth (B. Haller and J. Fuchs, unpublished results). The *trxA*7004 derivative of strain 71.18 (Heidecker et al., 1980), JF510, was constructed by phage P1 transduction and used to test the ability of filamentous bacteriophages M13 and f1 to form plaques.

E. coli strains were grown in liquid culture in Luria–Bertani medium (Miller, 1972) supplemented with glucose or in Davis–Mingioli minimal medium (Davis & Mingioli, 1950) supplemented with required components. When needed, ampicillin was added at final concentrations of 50 or 30 μg/mL to enriched or minimal medium, respectively. Plasmids pUC13 and pUC18 were used as cloning vectors. Phages M13mp2,

[1] Abbreviations: DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IPTG, isopropyl β-thiogalactoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; PEI, poly(ethylenimine); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

obtained from J. Messing, and wild-type f1, obtained from M. Russel, were used for tests of phage replication.

Construction of Hybrid Thioredoxins. Thioredoxin genes from *E. coli* (Lim et al., 1985a) and *Anabaena* sp. PCC 7119 (Lim et al., 1986) were isolated as previously described. The *Hin*dIII–*Ava*II fragment of insert pCJF4 which contains the N-terminal codons for *E. coli* thioredoxin and a corresponding *Ava*II–*Eco*RI fragment of insert pLGF13 which contains the C-terminal codons for *Anabaena* thioredoxin were isolated by gel electrophoresis. These restriction fragments were ligated with T4 DNA ligase. The large fragments of plasmid pUC13 and pUC18 were obtained by digestion with *Eco*RI and *Hin*dIII and gel purification. The isolated hybrid thioredoxin gene was joined to the plasmids with T4 DNA ligase. The resulting plasmids code for a hybrid thioredoxin gene having the N-terminal sequence (codons for approximately one-third of the protein) for the *E. coli* thioredoxin and the C-terminal sequence for *Anabaena* thioredoxin (E–A thioredoxin). By the same procedure, a *Hin*dIII–*Ava*II fragment of insert pLGF13 which contains the N-terminal codons for *Anabaena* thioredoxin and the *Ava*II–*Eco*RI fragment of pCJF4 which contains the C-terminal codons for *E. coli* thioredoxin were isolated and ligated to produce the reciprocal construct (A–E thioredoxin) (see Figure 1). Strains BH2012 or JF510 were transformed with these plasmids by the $CaCl_2$ procedure (Mandel & Higa, 1970) and plated on enriched medium containing ampicillin. The structures of plasmids encoding hybrid thioredoxins were confirmed by triple digestion with *Hin*dIII, *Eco*RI, and *Ava*II. The resulting plasmids, pCJL30 and pCJL40 with inserts in opposite orientations with respect to the *lac* promoter, code for the E–A thioredoxin. Plasmids pCJL50 and pCJL60 contain genes for the corresponding A–E thioredoxin.

Enzyme Assays. (*A*) *Thioredoxin Assays.* Three methods were used to detect and quantitate thioredoxin activity.

Method 1. Thioredoxin-catalyzed reduction of insulin by DTT was monitored as a turbidity increase at 650 nm (Holmgren, 1979). This method was used to detect thioredoxin activity in crude extracts of *E. coli*. Homogeneous thioredoxins from *Anabaena* or *E. coli* were used as standards. This method was also applied to estimate the ability of chemically reduced thioredoxins to function as a protein disulfide reductase.

Method 2. In the purification of thioredoxin from extracts of *E. coli* and for kinetic analysis, thioredoxin-catalyzed reduction of DTNB was monitored in the presence of *E. coli* thioredoxin reductase and NADPH [method 1 in Luthman and Holmgren (1982)].

Method 3. Thioredoxin activity was determined by monitoring insulin reduction in the presence of *E. coli* thioredoxin reductase and NADPH [method 4 in Luthman and Holmgren (1982)]. This method was used primarily to estimate antibody titers.

(*B*) *Ribonucleotide Reductase.* Ribonucleotide reductase activity was determined by measuring the production of [^{3}H]dCDP. The reaction mixture contained 80 mM HEPES buffer, pH 7.6, 60 μg of bovine serum albumin, 25 mM $MgCl_2$, 3 mM ATP, 1 mM DTT, 1.5 mM [^{3}H]CDP (approximately 10 000 cpm/nmol), and various concentrations of thioredoxin (0.5–10 μM) in a final volume of 0.05 mL. The reaction was initiated by addition of 2.5 μg of *E. coli* ribonucleotide reductase, and incubated at 37 °C. The reaction was stopped by boiling for 2 min. Deoxynucleotides were separated from ribonucleotides by chromatography on PEI–cellulose plates (Brinkmann Instruments, Inc., Westbury, NY) as previously described (Gleason & Holmgren, 1981). Labeled deoxynucleotide was eluted from the plate with 0.7 M $MgCl_2$ and added to PCS scintillation cocktail (Amersham Corp., Arlington, Heights, IL). Radioactivity was determined in a Searle Mark IV scintillation spectrometer.

Purification of Hybrid Thioredoxins. E. coli cells harboring thioredoxin genes were grown on enriched medium to an OD_{660} of 0.2, and IPTG was added to 0.1 mM final concentration. Growth was continued to late stationary phase for the strain containing the A–E thioredoxin hybrid and to late exponential phase for the strain containing the E–A hybrid. Cells were harvested by centrifugation and stored at −20 °C. Cells were disrupted by sonication. Thioredoxin was purified by ion-exchange (DEAE) chromatography using a 0–0.3 M NaCl gradient and G-75 Sephadex chromatography. Active fractions were pooled and concentrated by ultrafiltration or lyophilization. Protein was estimated from the absorbance at 280 and 310 nm (Kallis & Holmgren, 1980). Purity of the fractions was determined by polyacrylamide gel electrophoresis. Gels containing 15% acrylamide were run at pH 8.9 and stained with Coomassie blue (Davis, 1964).

Immunology. Polyclonal antibodies to *E. coli* and *Anabaena* thioredoxins were raised in rabbits. Two or one rabbit (respectively) was injected intramuscularly with approximately 300 μg of pure thioredoxin as an emulsion in complete Freund's adjuvant. The initial injection was followed 3 weeks later by another of thioredoxin in incomplete Freund's adjuvant and a third of thioredoxin in phosphate-buffered saline. The animals were bled 10–20 days after the last immunization, and antibodies specific for thioredoxin were detected in serum by their ability to inhibit thioredoxin-catalyzed insulin reduction (method 3) and by Ouchterlony immunodiffusion analysis. The antibodies were precipitated by 40% ammonium sulfate, and the precipitate was dialyzed against Tris-buffered saline. Protein was estimated by the Bradford method (Bradford, 1976).

The basic procedure for detecting thioredoxins by immunoblotting was adapted from that described by Tsang (Tsang et al., 1983). Native 15% polyacrylamide slab gels, containing 1.0 μg of purified thioredoxin per well, were run at 40 mA as described in Figure 3. The gels were cooled to 10 °C by circulating tap water. The resolved thioredoxins were transferred to nitrocellulose paper by horizontal electrophoresis for 3 h at 150 V with a water-cooled Hoefer Scientific "might small" transfer unit. After blocking with BSA, the paper was incubated at 25 °C for approximately 16 h with diluted antibody (1:2000) specific for either *E. coli* or *Anabaena* thioredoxin. Following buffer wash, the paper was incubated with diluted (1:1000) anti-rabbit IgG alkaline phosphatase conjugate. The reactive bands were visualized by alkaline phosphatase staining (Blake et al., 1984).

Kinetic Studies. Kinetic measurements of the reaction of thioredoxins with *E. coli* thioredoxin reductase were carried out according to method 2 using a Model 8450A Hewlett-Packard spectrophotometer at 25 °C. The kinetic parameters were obtained from unweighted least-squares analysis of Lineweaver–Burk plots. *E. coli* thioredoxin reductase concentration was estimated from the flavin absorbance at A_{460} (Thelander, 1967).

Fluorescence Measurements. Fluorescence spectra were recorded with a Shimadzu RF-510LC spectrometer. Measurements were made in a 1-cm path-length cell in a total volume of 1 mL. The exciting wavelength was 280 nm, and emission was recorded from 290 to 450 nm. The temperature was maintained at 30 °C by circulating water through the jacket of the cell holder. All thioredoxins were dissolved in

Table I: Plaque Formation of Phage on *Escherichia coli* Cells Containing Cloned Thioredoxin Genes[a]

	thioredoxin type (plasmid)				
phage	*E. coli* (pCJF4)	none (pUC13)	*Anabaena* (pLGF13)	A–E (pCJL50,60)	E–A (pCJL30,40)
T7	+	–	–	+	–
T7tas1	+	–	+	+	+
M13mp2	+	–	+	+	–
f1	+	–	+[b]	+	–

[a] (+) Growth; (–) no growth. [b] Wild-type f1 phage formed very tiny, unclear plaques on strains containing plasmid pLGF13 (Lim et al., 1986).

50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. All buffers were thoroughly degassed before use. The A_{280} of the samples was between 0.03 and 0.05. Thioredoxins were reduced by addition of 1 mM DTT (final concentration) to the cuvette. Thioredoxins were denatured by incubation for 15 min in 5 M guanidine hydrochloride in the above buffer. Solvent blanks were run and subtracted from the sample spectra. The spectra were not corrected for monochromator and detector responses. Relative quantum yields were calculated by using L-tryptophan in buffer as a standard (Q = 0.144; Wiget & Luisi, 1978).

Results

Biological Characterization of Hybrid Thioredoxins in E. coli. *E. coli trxA*$^-$ mutant strains exhibit reduced colony size when plated on enriched medium. It had previously been shown that large colony size was restored in transformants containing plasmid pLGF13 which codes for *Anabaena* thioredoxin (Lim et al., 1986) or pCJF4 which codes for *E. coli* thioredoxin. In like manner, both hybrid-type plasmids (pCJL50, pCJL60 and pCJL30, pCJL40) restored normal growth in the mutant strain. The plasmids coding for hybrid thioredoxins are also able to complement a *gshA trxA* double mutant, BH5262, which otherwise requires glutathione for growth. *E. coli* BH2012 (*trx*A *met*E) is unable to use methionine sulfoxide to satisfy its methionine requirement. However, BH2012 (pCJL50 and 60) and BH2012 (pCJL30 and 40) will grow on methionine sulfoxide. These results indicate that both hybrid thioredoxins function in vivo. However, since these hybrid thioredoxin genes are present on a high copy number plasmid, the data do not confirm that the hybrid proteins function as efficiently as *E. coli* thioredoxin.

E. coli BH2012 will not support the growth of T7 bacteriophage because the host thioredoxin is an essential accessory protein for the viral DNA polymerase (Modrich & Richardson, 1975). This same strain containing plasmids pCJL50 or pCJL60, coding for the A–E hybrid thioredoxin, will give rise to plaques after infection. In contrast, strains containing plasmids pCJL30 or pCJL40, encoding the E–A hybrid, do not support phage growth. It had previously been found that when high titers of T7 phage were plated on strain BH2012 containing plasmid pLGF13 which codes for *Anabaena* thioredoxin, mutant phage which will replicate in this strain could be isolated. An isolate of this phage has been designated T7tasl for "thioredoxin altered specificity" which presumably has a mutation in the T7 gene 5 (Lim et al., 1986). This mutant phage was able to replicate in the presence of either hybrid thioredoxin. As seen in Table I, the A–E hybrid is similar to *E. coli* thioredoxin, and the E–A hybrid resembles *Anabaena* thioredoxin with respect to support of T7 replication.

E. coli thioredoxin is also required for filamentous phage assembly. Strains JF510 (pCJL50 or 60) containing the A–E hybrid thioredoxin could support the growth of these viruses as well as the wild-type *E. coli*, whereas strains JF510 (pCJL30 or 40) expressing the E–A hybrid thioredoxin could not. Strain JF510 containing either *E. coli* or *Anabaena* thioredoxin can support both M13 and f1 replication (see Table I).

Table II: Reduction of Thioredoxins with *Escherichia coli* Thioredoxin Reductase[a]

thioredoxin	K_m (μM)	k_{cat} (min^{-1})	catalytic efficiency (k_{cat}/K_m)
E. coli	2.0	2330	1165
Anabaena	17	2330	137
Anabaena–E. coli	1.0	3105	3105
E. coli–Anabaena	100	1750	18

[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 0.5 mL. Thioredoxins were added in a concentration range from 0.08 to 10 μM. The reaction was initiated by adding *E. coli* thioredoxin reductase at a concentration of 36 nM as determined by the flavin absorbance at A_{460} (Thelander, 1967). The increase in A_{412} was monitored at 25 °C, and activities were calculated by method 1 in Luthman and Holmgren (1982).

Table III: Ability of Hybrid Thioredoxins To Serve as Reducing Agents for *E. coli* Ribonucleotide Reductase[a]

thioredoxin	K_m (μM)	k_{cat} (min^{-1})	catalytic efficiency (k_{cat}/K_m)
E. coli	2.0	120	60
Anabaena	2.0	120	60
Anabaena–E. coli	1.0	160	160
E. coli–Anabaena	3.3	48	15

[a] CDP reductase activity was determined in the presence of 1 mM DTT as described under Materials and Methods.

Isolation and Characterization of the Hybrid Thioredoxins. Hybrid thioredoxins were purified to approximately 95% homogeneity by the procedure previously described for *E. coli* thioredoxin. Insulin precipitation activity (method 1) in extracts of strains containing the E–A hybrid protein was low and variable in stationary cells, suggesting that the protein is unstable in vivo. The yield of E–A thioredoxin was 10-fold lower than the corresponding A–E hybrid. On native, alkaline polyacrylamide gels, the hybrid thioredoxins exhibit intermediate mobilities between the acidic *E. coli* thioredoxin (isoelectric point = 4.5) and the less acidic *Anabaena* thioredoxin (isoelectric point = 6.5) (see Figure 3).

Both hybrid thioredoxins serve as substrates for *E. coli* thioredoxin reductase which measures the ability of the thioredoxin to be bound by the reductase and accept electrons. However, as shown in Table II, the A–E hybrid thioredoxin exhibits a much higher catalytic efficiency than even the natural substrate, *E. coli* thioredoxin. The E–A construct, on the other hand, has a much higher K_m than either parent molecule, indicating poor interaction with the *E. coli* thioredoxin reductase.

In contrast to the thioredoxin reductase, *E. coli* ribonucleotide reductase seems to have very low specificity for thioredoxin and interacts equally well with both *E. coli* and *Anabaena* thioredoxins as illustrated in Table III. This reaction measures the efficiency of H transfer by reduced

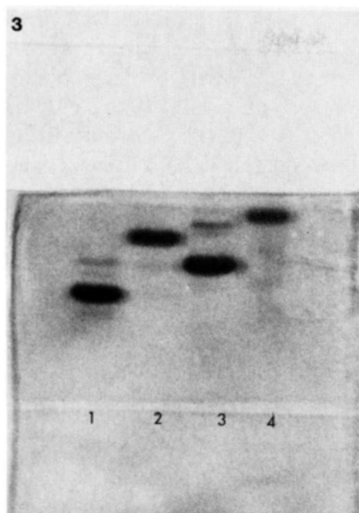


FIGURE 3: Separation of thioredoxins by native polyacrylamide gel electrophoresis. Thioredoxins were purified from extracts of *E. coli* strains containing the following plasmids: pCJF4 (*E. coli* thioredoxin), pLGF13 (*Anabaena* thioredoxin), pCJL30 (E–A thioredoxin), and pCJL50 (A–E thioredoxin). Thioredoxins were purified by chromatography on DEAE-cellulose and Sephadex G-75. For *Anabaena* thioredoxin, an additional step, utilizing PBE94 chromatofocusing gel, was added to obtain homogeneous preparations. Protein (approximately 5 μg) of each type was loaded onto a native 15% polyacrylamide gel and run at pH 8.6. Lane 1, *E. coli* thioredoxin; lane 2, A–E thioredoxin, lane 3, E–A thioredoxin; lane 4, *Anabaena* thioredoxin.

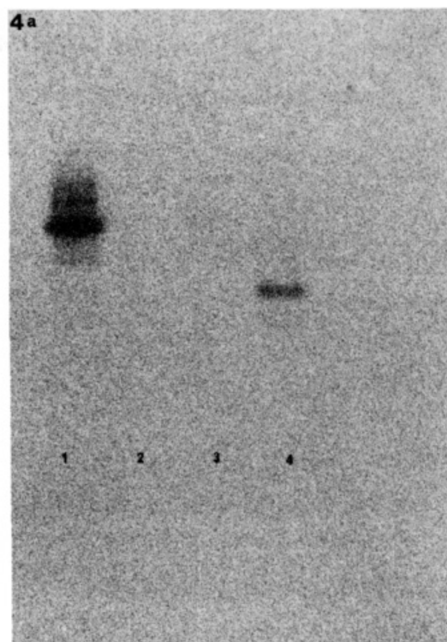
Table IV: Reaction of Thioredoxins with Antibodies[a]

thioredoxin	addition: none	addition: anti-*E. coli* thioredoxin	addition: anti-*Anabaena* thioredoxin
E. coli	2.64[b]	0.23[b]	1.96[b]
Anabaena	0.91	0.51	0.36
Anabaena–E. coli	3.95	1.49	3.27
E. coli–Anabaena	0.21	0.23	0.09

[a] Activity was determined by measuring the thioredoxin-catalyzed reduction of insulin in the presence of *E. coli* thioredoxin reductase (1.4 nM) and NADPH (method 3). Reaction vessels were incubated at 37 °C for 20 min to allow sufficient time for thioredoxin turnover. Partially purified antibody was added at a final concentration of 25 μg of protein per reaction (anti-*E. coli* thioredoxin antibody) or 15 μg of protein per reaction (anti-*Anabaena* thioredoxin antibody). [b] Nanomoles of NADPH oxidized per minute per microgram of thioredoxin.

thioredoxin. The A–E hybrid is superior to the natural thioredoxins in this reaction as shown by a lower K_m and a slightly higher turnover number. The E–A construct binds poorly to the reductase, and the turnover is 2.5-fold slower than the parent thioredoxins, making it a relatively inefficient H-transfer agent.

Immunological Results. Polyclonal antibodies to thioredoxin raised in rabbits will inhibit the thioredoxin-catalyzed reduction of insulin. The antibodies are relatively species specific and exhibit only moderate cross-reactivity with other thioredoxins when assayed by method 3 (see Table IV). The protein disulfide reductase activity of the hybrid thioredoxins is also inhibited by antibodies. The A–E hybrid thioredoxin is recognized by antibody to *E. coli* thioredoxin, but not by antibody to *Anabaena* thioredoxin. The E–A thioredoxin has antigenic sites for *Anabaena* thioredoxin antibodies. The data indicate that the thioredoxin antigenic determinants are located primarily in the C-terminal portion of the polypeptide chain. This conclusion was further confirmed by immunoblotting



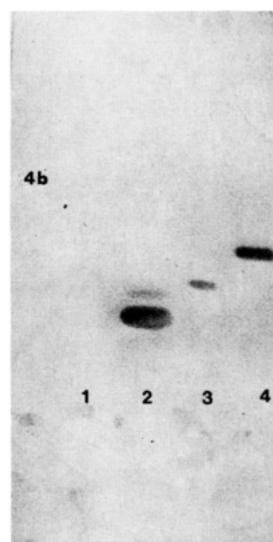


FIGURE 4: Western immunoblot analysis of thioredoxins. Approximately 1 μg of purified thioredoxins was separated on native 15% gels as described in Figure 3. The proteins were then transferred to nitrocellulose paper and reacted with antibody to *Anabaena* thioredoxin (a) or antibody to *E. coli* thioredoxin (b). Reactive conjugates were visualized by alkaline phosphatase staining as described under Materials and Methods. (a) Cross-reaction with anti-*Anabaena* thioredoxin. Lane 1, *Anabaena* thioredoxin; lane 2, *E. coli* thioredoxin; lane 3, A–E thioredoxin; lane 4, E–A thioredoxin. (b) Cross-reaction with anti-*E. coli* thioredoxin. Lane 1, *Anabaena* thioredoxin; lane 2, *E. coli* thioredoxin; lane 3, E–A thioredoxin; lane 4, A–E thioredoxin.

which is illustrated in Figure 4. By this procedure, antibodies to *Anabaena* thioredoxin are seen to bind to *Anabaena* thioredoxin and the E–A hybrid, but not to *E. coli* or A–E hybrid thioredoxins. Antibodies to *E. coli* thioredoxin react most strongly with *E. coli* and A–E thioredoxins.

Fluorescence Spectra. Both *E. coli* and *Anabaena* thioredoxins have typical tryptophan-dominated fluorescence spectra which show a 2–3-fold increase in intensity on reduction [data now shown; see Holmgren (1972) and Gleason and Holmgren (1981)]. The emission spectra of the E–A hybrid thioredoxin are similar to those of *Anabaena* thioredoxin (data not shown). The Trp fluorescence in the A–E hybrid protein is considerably more quenched than in the parent molecules, and the resulting spectrum of oxidized A–E

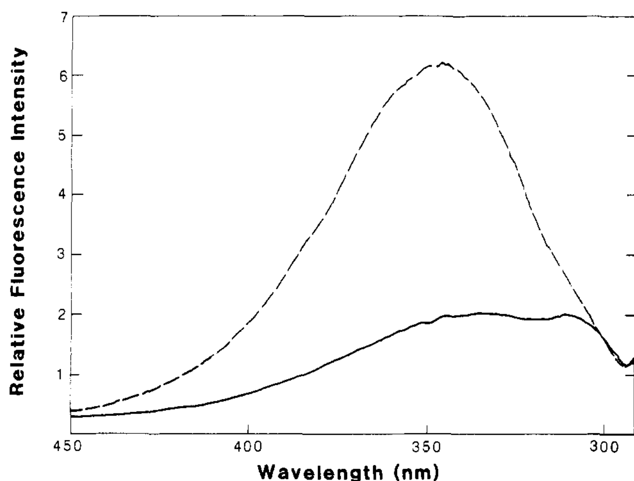


FIGURE 5: Fluorescence emission spectra of A–E hybrid thioredoxin. Thioredoxin was dissolved in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA. Spectra were recorded after excitation at 280 nm. (—) Oxidized A–E thioredoxin; (---) reduced A–E thioredoxin (1 mM DTT).

Table V: Fluorescence Properties of Thioredoxins[a]

thioredoxin	quantum yield ($\times 10^2$)		
	oxidized	reduced	denatured
E. coli	14 (345)	43 (345)	40 (360)
Anabaena	19 (340)	36 (345)	45 (360)
Anabaena–E. coli	7 (345)	21 (345)	34 (355)
E. coli–Anabaena	22 (350)	37 (350)	36 (360)

[a] The excitation wavelength was 280 nm. Spectra were run at pH 7.0 in 50 mM potassium phosphate buffer at 30 °C. Thioredoxins were reduced with 1 mM DTT. Numbers in parentheses indicate the wavelength of maximum emission. Quantum yields were calculated on the basis of Q of tryptophan in water = 0.144 (Wiget & Luisi, 1978).

protein exhibits a maximum at 310 nm and a broad plateau centered at approximately 345 nm as seen in Figure 5. A 3-fold increase in intensity is recorded after reduction. The relative quantum yields for oxidized, reduced, and denatured thioredoxins are shown in Table V. All thioredoxins show the expected red shift in emission maximum on unfolding.

Discussion

Previous analysis of *Anabaena* thioredoxin had shown that it will interact with *E. coli* proteins both in vivo and in vitro. The algal thioredoxin is a good reducing agent for *E. coli* ribonucleotide reductase and is reduced by *E. coli* thioredoxin reductase, although with a 10-fold higher K_m (Gleason, 1986). The *Anabaena* thioredoxin gene, when cloned into thioredoxin-lacking strains of *E. coli*, will restore the wild-type phenotype except for replication of some phage (Lim et al., 1986). The *Anabaena–E. coli* hybrid thioredoxins also appear to be fully functional in vivo. Thioredoxin-deficient bacteria which express hybrid thioredoxins grow on methionine sulfoxide, indicating that both hybrid proteins are reduced and can serve as reducing agents for methionine sulfoxide reductase. Hybrid thioredoxins also apparently serve as cofactors for an *E. coli* function that is deficient in the glutathione–thioredoxin double mutants.

Anabaena thioredoxin will not form an active complex with the T7 phage gene 5 protein, and thioredoxin-deficient strains which express *Anabaena* thioredoxin will not support phage growth. Although both *Anabaena* and *E. coli* thioredoxins have the same redox active-site sequence, interaction with the gene 5 protein requires the recognition of additional amino acids in the molecule. The failure of *Anabaena* thioredoxin to effectively interact with the viral protein has been ascribed to subtle changes in the C-terminal sequences such as a serine in position 74 in place of the glycine in *E. coli* thioredoxin as noted in Figure 1 (Lim et al., 1986). This prediction is supported by the results obtained with hybrid thioredoxins. The A–E hybrid protein, with the C-terminal sequence of *E. coli* thioredoxin, can support T7 phage growth. The E–A construct cannot. It may be that higher levels of this hybrid thioredoxin are required to support viral replication than are produced by these transformed strains especially since the E–A hybrid thioredoxin appears to be unstable and may be rapidly degraded, particularly in stationary phase cultures. However, this thioredoxin can support the growth of the T7tas phage which implies that sufficient E–A thioredoxin is produced in the cell during exponential growth and that it does not form an active complex with the wild-type gene 5 protein due to a structural difference in the C-terminal sequence. Recent studies on mutant thioredoxins by Huber and co-workers support our conclusions (Huber et al., 1986). These workers reported that amino acid changes in the active-site sequence, i.e., proline-34 to serine or cysteine-32 to serine, had little effect on phage growth. In contrast, when glycine-74 was altered to aspartate, or glycine-92 to serine, the plating efficiency of T7 in the resulting strains was greatly reduced. The data on mutant and hybrid thioredoxins indicate that subtle changes in the C-terminal sequences preclude efficient interaction with the gene 5 protein while the N-terminal residues do not have a direct role in this interaction and are inconsequential. A similar conclusion can be drawn from the results obtained on filamentous phage replication. *E. coli* strains containing *Anabaena* thioredoxin will support M13 and f1 growth, although rather poorly. Replacement of the *Anabaena* with the *E. coli* C-terminal sequence in the strains containing the A–E hybrid thioredoxin results in vigorous phage production. Strains containing the E–A hybrid thioredoxin do not support these viruses at all. It appears that the *Anabaena* C-terminal sequence combined with conformational strains in this hybrid renders it completely inactive, at least at current levels of expression.

Both hybrid thioredoxins are reduced in vivo, suggesting efficient interaction with *E. coli* thioredoxin reductase. However, in mutants lacking thioredoxin reductase, thioredoxin is still reduced by some alternate mechanism (Fuchs, 1977). The kinetics of reduction of the hybrid thioredoxins, as shown in Table II, illustrate an interesting dichotomy. Comparing the catalytic efficiency of *E. coli* and A–E hybrid thioredoxins, the A–E hybrid protein is a slightly superior substrate for the reductase. In contrast, the E–A hybrid is inferior even to *Anabaena* thioredoxin. These differences are mainly due to changes in the K_m's. The less efficient binding of *Anabaena* thioredoxin to *E. coli* reductase has been attributed to altered residues in the active-site region, i.e., proline-30 in place of glutamic acid, arginine-36 in place of lysine, and substitution of a serine at position 74 in place of glycine in *E. coli* thioredoxin (see Figure 1). In the A–E hybrid thioredoxin, lysine-36 and glycine-74 are restored, but proline-30 remains. The kinetic data suggest that the presence of this residue in the hybrid may actually enhance binding to the reductase by changing the configuration of the protein in the active-site region. The higher K_m of the E–A hybrid thioredoxin cannot simply be a function of single-residue replacements in the C-terminal sequence since it is a poorer substrate than *Anabaena* thioredoxin. Even though it appears that the N-terminal sequence does not actively participate in thioredoxin–protein interactions, it must still play a role in maintaining the conformational integrity of the active site, which is obviously

impaired in the E–A hybrid molecule. These data are supported by the results obtained with *E. coli* ribonucleotide reductase. Although less striking than the above, the E–A hybrid is still the least efficient H donor in this reaction, while the A–E hybrid functions better than either parent molecule.

The rabbit polyclonal antibodies to *E. coli* and *Anabaena* thioredoxins are species specific as shown by the immunoblots in Figure 4, despite the homology between the proteins. Reaction of the hybrid thioredoxins to these antibodies indicates that the antigenic determinants for thioredoxin are located in the C-terminal two-thirds of the molecule. Similar findings had previously been reported for *E. coli* thioredoxin (Holmgren et al., 1981). Since antibodies apparently recognize unique sequences in the native, folded protein (Benjamin et al., 1984), our data indicate that the hybrid thioredoxins maintain the conformational integrity of their parent proteins at antigenic sites.

A sensitive indicator of the conformation in the active-site region of thioredoxin is the fluorescence emission of tryptophan-28. The fluorescence intensity of this residue is quenched in oxidized thioredoxin, presumably because of its proximity to the active-site disulfide. On reduction, there is a 2–3-fold increase in intensity (Holmgren, 1972). *Anabaena* thioredoxin and the hybrid proteins all retain this Trp in the same position and exhibit a similar increase in intensity when reduced. However, the Trp-28 fluorescence in the A–E hybrid is considerably more quenched than in the parent molecules (see Figure 5). The second peak in the spectrum at 310 nm may be due to tyrosine emission and appears as a shoulder on the Trp emission in the spectra of the other thioredoxins. Although the intensity increases on reduction of the A–E hybrid, the quantum yield is still depressed as seen from Table V. The E–A hybrid exhibits the opposite behavior, although not as pronounced. The fluorescence data confirm that the conformation of the active site has undergone a subtle alteration in the hybrid thioredoxins. The increase in quantum yield of the E–A hybrid may indicate that Trp-28 is in a less polar environment or positioned further away from the quenching group. Conversely, the decreased quantum yield in the A–E hybrid could be explained by a more "open" active-site region in which the Trp-28 is exposed to water or positioned closer to the quenching residue. Since the quantum yield of the reduced form is still low, the data suggest the latter possibility is more likely and that some residue other than the cystine disulfide contributes to the Trp-28 quenching observed in most thioredoxins.

One goal of protein engineering is to elucidate enzyme reaction mechanisms by introducing specific amino acid changes into the protein molecule. The problem is to determine which residues to alter so that catalytic activity is perturbed, but the overall conformational integrity of the molecule is maintained. Loss of activity on alteration of a particular amino acid residue does not necessarily confirm its role in catalysis. This problem may be particularly acute when investigating protein–protein interactions since large sections of the molecule are involved. A comparison of amino acid sequences of bacterial thioredoxins suggests that in addition to the active-center disulfide, other highly conserved residues may play a role in electron transport. Variable residues will maintain the conformation of the molecule and presumably "recognize" other proteins which are specific to thioredoxin interactions in a particular organism. Our analysis of the reactivity of hybrid thioredoxins corroborates the conclusions of Bränden and co-workers from model-building studies. These workers proposed that certain hydrophobic sequences in the C-terminal domains of thioredoxin are involved in specific protein interactions (Bränden et al., 1983). Our results show that for *E. coli* thioredoxin, the C-terminal portion of the molecule maintains the native conformation despite extensive changes in the N-terminal third of the protein and contains the major domains which recognize and interact with other proteins. Replacing the N-terminal domain with *Anabaena* residues as in the A–E hybrid does not affect activity in the cell or in vitro in any major way. In fact, this chimera is actually a superior electron-transfer agent due to a better "fit" between the two domains of the molecule. The opposite construction, the E–A thioredoxin, also appears to be effective in some reactions in vivo and is still a substrate for *E. coli* thioredoxin reductase and ribonucleotide reductase, although a rather inefficient one. Since its K_m in this reaction is even higher than that of *Anabaena* thioredoxin, it must be concluded that the two parts of the molecule coordinate poorly and that the N-terminal sequence plays a subtle but significant role in maintaining the active-site conformation.

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Effect of Inhibitors of *S*-Adenosylmethionine Decarboxylase on Polyamine Content and Growth of L1210 Cells[†]

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ABSTRACT: Analogues of *S*-adenosylmethionine that were designed as inhibitors of *S*-adenosylmethionine decarboxylase were tested for their abilities to inhibit the purified enzyme from rat prostate. The most potent inhibitors were 5′-deoxy-5′-[*N*-methyl-*N*-[2-(aminooxy)ethyl]amino]adenosine (MAOEA) and 5′-deoxy-5′-[*N*-methyl-*N*-(3-hydrazinopropyl)amino]adenosine (MHZPA), which had I_{50} values of 400 nM and 70 nM, respectively, when added directly to the assay medium under standard conditions. These compounds were irreversible inactivators of the enzyme, and more than 95% of the activity was lost within 15 min of exposure to 5 μM MAOEA or 0.5 μM MHZPA. Both inhibitors led to a large reduction in the content of decarboxylated *S*-adenosylmethionine in L1210 cells and to a substantial decrease in the production of 5′-(methylthio)adenosine by these cells. These results are consistent with their bringing about an inhibition of *S*-adenosylmethionine decarboxylase activity in the cell which leads to a reduction in the synthesis of spermidine and spermine. Analysis of the polyamine content in L1210 cells exposed to 100 μM MAOEA or 50 μM MHZPA showed that this was the case and that putrescine levels were greatly increased while spermidine and spermine content declined. The combined application of 100 μM MAOEA and 5 mM α-(difluoromethyl)ornithine (an ornithine decarboxylase inhibitor) to L1210 cells completely prevented the synthesis of putrescine, spermidine, and spermine for up to 48 h. The reduction in polyamine content brought about by MHZPA or MAOEA could be partially prevented by the addition of decarboxylated *S*-adenosylmethionine to the culture medium. These inhibitors also brought about an inhibition of cell growth which could be reversed by the addition of spermidine. These results indicate that inhibitors of *S*-adenosylmethionine decarboxylase block cell growth by means of their inhibition of the production of spermidine and that putrescine cannot satisfy the requirement for spermidine in the growth of L1210 cells. They also demonstrate that analogues of *S*-adenosylmethionine are taken up by some mammalian cells and can influence polyamine metabolism. Such compounds have considerable potential as therapeutic agents and for studies of the function of polyamines.

Recent results with inhibitors of ornithine decarboxylase suggest that the polyamine biosynthetic pathway is likely to be an important target for the design of chemotherapeutic agents (Pegg & McCann, 1982; Jänne et al., 1983; Sjoerdsma & Schechter, 1984; Porter & Sufrin, 1986; Pegg, 1986; Schechter et al., 1987). Such inhibitors lead to a virtually complete depletion of spermidine and putrescine but have little effect on spermine and produce an immense increase in the cellular content of decarboxylated *S*-adenosylmethionine. These changes lead to a reduction of cellular growth and alterations in cellular differentiation. The therapeutic value of ornithine decarboxylase inhibitors suggests that inhibitors of other steps in the polyamine biosynthetic pathway may also have pharmacological potential. The combination of inhibitors for different reactions in this pathway may have synergistic effects. Also, inhibitors of each of the steps would permit more

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