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Cloning, Expression, and Characterization of the *Anabaena* Thioredoxin Gene in *Escherichia coli*

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The gene encoding thioredoxin in *Anabaena* sp. strain PCC 7119 was cloned in *Escherichia coli* based on the strategy that similarity between the two thioredoxins would be reflected both in the gene sequence and in functional cross-reactivity. DNA restriction fragments containing the *Anabaena* thioredoxin gene were identified by heterologous hybridization to the *E. coli* thioredoxin gene following Southern transfer, ligated with pUC13, and used to transform an *E. coli* strain lacking functional thioredoxin. Transformants that complemented the *trxA* mutation in *E. coli* were identified by increased colony size and confirmed by enzyme assay. Expression of the cloned *Anabaena* thioredoxin gene in *E. coli* was substantiated by subsequent purification and characterization of the algal protein from *E. coli*. The amino acid sequence derived from the DNA sequence of the *Anabaena* gene was identical to the known amino acid sequence of *Anabaena* thioredoxin. The *E. coli* strains which expressed *Anabaena* thioredoxin complemented the TrxA$^-$ phenotype in every respect except that they did not support bacteriophage T7 growth and had somewhat decreased ability to support bacteriophages M13 and f1.

Thioredoxin is a small (M_r, ~12,000) redox protein with an active center cystine disulfide or dithiol. This protein participates in a number of oxidation-reduction and regulatory functions in various cells. Thioredoxin can act as a reducing agent for ribonucleotide reductase and as a general protein disulfide reductase in both bacterial and mammalian cells (see reference 15 for a review). In addition, thioredoxin is required for the replication or assembly of some bacterial viruses. T7 DNA polymerase (gene 5 protein) is active only when it forms a complex with the host thioredoxin (26). *Escherichia coli* thioredoxin is also essential for the assembly of M13 and f1 viruses, although the role of the protein in these processes is unknown (19, 29).

In photosynthetic organisms, multiple thioredoxins have been reported to occur in both chloroplasts and cytoplasm. Thioredoxins from chloroplasts regulate the activity of enzymes of carbon dioxide fixation such as fructose bisphosphatase (33) and glucose-6-phosphate dehydrogenase (1), presumably by reducing essential sulfhydryl groups of these enzymes. The cyanobacteria are the simplest organisms with an oxygen-evolving photosynthetic system and have been used as a model for investigation of chloroplast function. In the cyanobacterium *Anabaena* sp. strain PCC 7119, thioredoxin has been shown to be a reducing agent for ribonucleotide reductase and an effective protein disulfide reductase (10). The primary structure of thioredoxin from this photosynthetic procaryote is approximately 50% homologous to the thioredoxin from *E. coli* and *Corynebacterium nephridii* (11). A recent report also indicates that at least one of the spinach chloroplast thioredoxins is closely related to the algal protein (21).

By taking advantage of this high degree of primary structural homology between *Anabaena* and *E. coli* thioredoxins, we successfully cloned the gene encoding *Anabaena* thioredoxin by complementation of an *E. coli* mutant lacking thioredoxin. The thioredoxin produced in *E. coli* by the cloned gene was purified and shown to be identical to *Anabaena* thioredoxin. The cloned gene was sequenced and found to have an open reading frame that would encode a protein identical to *Anabaena* thioredoxin. Cloning of the *Anabaena* thioredoxin gene in *E. coli* made it possible to further analyze the in vivo structural requirements for thioredoxin activity in the bacterial system. In addition, it will now be possible to investigate the in vivo role of thioredoxin in an oxygen-evolving photosynthetic organism and to use the protein produced from the cloned gene for thioredoxin structure-function studies.

MATERIALS AND METHODS

Materials. All restriction endonucleases, T4 DNA ligase, bacterial alkaline phosphatase, and *E. coli* DNA polymerase I Klenow fragment were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Indianapolis, Ind. All enzymes were used as specified by the manufacturer. Dithiothreitol, 5,5′-dithiobis(1-nitrobenzoic acid) (DTNB), NADPH, bovine insulin, lysozyme, RNase A, subtilisin, acrylamide, and imidazole were from Sigma Chemical Co., St. Louis, Mo. DEAE-Sephacel, Sephadex G-50, chromatofocusing gel PBE 94, and polybuffer were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from United States Biochemical Corp., Cleveland, Ohio. YM-5 ultrafiltration membranes (molecular weight cutoff, 5,000) were obtained from Amicon Corp., Lexington, Mass. Sodium dodecyl sulfate (SDS) and Bradford reagent were purchased from Bio-Rad Laboratories, Richmond, Calif. Spectropore 3 dialysis membrane and reagent grade organic solvents were from Fisher Scientific Co., Pittsburgh, Pa. [α-^{32}P]dCTP was from New England Nuclear Corp., Boston, Mass. [^{35}S]dATP was obtained from Amersham Corp., Arlington Heights, Ill. A homogeneous preparation of *E. coli* thioredoxin was a generous gift of Arne Holmgren, Karolinska Institute, Stockholm, Sweden.

Bacterial strains and phages. Strain BH2012, which is *E. coli* K-12 F$^-$ *araD139*? *galU galK hsr rpsL metA46 argH1 trxA7004 ilvC*::Tn*5*, was used as a recipient for transforma-

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tion. As previously reported (18), TrxA$^+$ derivatives of BH2012 can be identified by increased colony size on rich medium. BH2012 is unable to use methionine sulfoxide as a methionine source, whereas an isogenic TrxA$^+$ derivative can. This phenotype was used as a test of the ability of a foreign thioredoxin to serve as a cofactor for methionine sulfoxide reductase in *E. coli*. Derivatives of BH2012 were also used to test for growth of bacteriophage T7. Strain BH5262, which is *E. coli* K-12 F$^-$ *araD139? galU galK hsr rpsL argH1 trxA7004 gshA 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 Gsh$^+$ or TrxA$^+$ do not require glutathione for growth (B. Haller and J. A. Fuchs, unpublished data). The *trxA7004* derivative of strain 71.18 (13), 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 supplemented with glucose. When needed, ampicillin was added to the medium at a final concentration of 50 μg/ml. Plasmid pUC13 was used as a cloning vector. Phages M13mp2, obtained from J. Messing, and wild-type f1, obtained from M. Russel, were used for infection tests.

Growth of *Anabaena* sp. and extraction of genomic DNA. *Anabaena* sp. strain PCC 7119 (ATCC 29151) was grown in the presence of 5% CO_2 in air and harvested during the late exponential growth phase as previously described (9). Frozen cells were thawed and suspended in 50 ml of buffer containing 50 mM each Tris hydrochloride (pH 8.5), EDTA, and NaCl. Lysozyme was added to a final concentration of 2 mg/ml (wt/vol). The suspension was incubated at 37°C for 1 h. Microscopic examination indicated that over 90% of the cells were lysed. Genomic DNA was then extracted from the lysate as described by Mevarech et al. (25) for the related strain *Anabaena* sp. strain 7120.

Selection of restriction fragments by DNA-DNA hybridization. *Anabaena* sp. strain PCC 7119 genomic DNA was digested with endonuclease *Eco*RI or *Hin*dIII, partially digested with endonuclease *Hpa*II, and electrophoresed in a 1% agarose gel. The digested DNA was transferred to nitrocellulose and hybridized (23) to ^{32}P-labeled probe. The 460-base-pair (bp) *Hin*dIII-*Hin*cII insert fragment of pCJF5 (18), which contains the *E. coli* thioredoxin gene, was used as a probe. The DNA fragment was isolated from an agarose gel and ligated to generate polymers of the fragment that were nick translated in the presence of (α-^{32}P)dCTP. The labeled DNA probe was separated from unincorporated nucleotides on a Bio-Gel A 1.5-m column equilibrated with buffer containing 20 mM Tris hydrochloride (pH 8.0), 50 mM NaCl, and 1 mM EDTA. DNA-DNA hybridization was performed at 55°C overnight in hybridization solution (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 10 mM EDTA, 5× Denhardt solution, 0.5% SDS, 100 μg of denatured herring sperm DNA per ml) described by Maniatis et al. (23). The nitrocellulose filter was rinsed in a solution of 2× SSC–0.5% SDS and then washed in a solution of 0.1× SSC–0.5% SDS at room temperature for 1 h. Autoradiography was at −70°C on Kodak XAR-5 film with an intensifying screen.

Cloning of *Anabaena* gene encoding thioredoxin. Plasmid pUC13 was digested with *Hin*dIII, *Eco*RI, or *Acc*I and treated with bacterial alkaline phosphatase. *Anabaena* sp. strain PCC 7119 genomic DNA fragments of the size identified by DNA-DNA hybridization were removed from the 1% agarose gel by electroelution (23) followed by phenol extraction and ethanol precipitation. The isolated DNA fragments were ligated to the digested pUC13 with T4 DNA ligase. In parallel experiments, *Eco*RI-, *Hin*dIII-, or *Hpa*II-digested genomic DNA fragments were ligated to *Eco*RI-, *Hin*dIII-, or *Acc*I-digested pUC13, respectively. All ligation reactions were incubated at 14°C overnight. Strain BH2012 was transformed with the ligation mixtures by the $CaCl_2$ procedure (22) and plated on enriched plates containing ampicillin. TrxA$^+$ colonies were identified by increased colony size.

Thioredoxin assays. Three assays were used to detect thioredoxin activity. **(i) Method 1.** In crude extracts, thioredoxin-catalyzed reduction of insulin by dithiothreitol was monitored as a turbidity increase at 650 nm (14). The time required to initiate precipitation was used as an estimate of thioredoxin activity in the extracts. Homogeneous thioredoxin from *Anabaena* sp. strain PCC 7119 was purified by the previously published procedure (10) and used as a standard.

(ii) Method 2. In the purification of thioredoxin from extracts of *E. coli*, we determined thioredoxin activity by monitoring insulin reduction in the presence of *E. coli* thioredoxin reductase and NADPH (method 4 in reference 20).

(iii) Method 3. For kinetic analysis, thioredoxin-catalyzed reduction of DTNB was monitored in the presence of *E. coli* thioredoxin reductase and NADPH (method 1 in reference 20).

Purification of *Anabaena* thioredoxin from *E. coli*. Approximately 100 g of frozen *E. coli* cells (grown to an optical density at 660 nm of ≅0.2 in rich medium before IPTG was added to 0.1 mM and then to late stationary phase) was thawed and treated as described for *Anabaena* sp. (10) except that the extract was heated to 65 instead of 70°C. Fractions from the DEAE column containing thioredoxin activity eluted at approximately 0.1 M NaCl in the same position as previously reported for *Anabaena* thioredoxin. Active fractions from the subsequent Sephadex G-50 column were pooled and dialyzed for 24 h against several liters of 25 mM imidazole hydrochloride buffer, pH 7.4. The dialyzed fraction was then loaded onto a column of PBE 94 chromatofocusing gel (25 by 1.6 cm) and eluted with the same buffer. A pH gradient was then produced by eluting the column with polybuffer 74 (1:8 dilution) at pH 4.0. Thioredoxin activity was found in fractions at a pH of approximately 6.5. The active fractions were pooled and concentrated by ultrafiltration.

Purity of the fractions and identity to authentic *Anabaena* thioredoxin were determined by polyacrylamide gel electrophoresis. Gels containing 15% acrylamide were run at pH 8.9 (6). SDS-polyacrylamide gel electrophoresis with 15% acrylamide was performed as described by Weber et al. (32). Protein was determined by the Bradford method (2).

DNA sequence determination. DNA restriction fragments were subcloned into M13mp18 and M13mp19 (28) and sequenced by the dideoxy chain termination method (30) with a synthetic 17-base universal primer.

RESULTS

Cloning of the *Anabaena* thioredoxin gene. The *E. coli* DNA probe hybridized to 1.1- and 1.2-kilobase (kb) *Hpa*II fragments, a 3.7-kb *Eco*RI fragment, and an approximately 9-kb *Hin*dIII fragment of *Anabaena* genomic DNA (data not shown). The appropriate-size fragments were eluted from a gel, ligated to pUC13, and used to transform BH2012 as described in Materials and Methods. Twelve large colonies

TABLE 1. Thioredoxin activity of heat-treated extracts of *E. coli* BH2012 containing various plasmids after IPTG induction

Strain	Thioredoxin activity[a]
MC1061[b]	2.8
BH2012(pUC13)	0
BH2012(pLGF6)	53.7
BH2012(pLGF8)	21.1
BH2012(pLGF13)	125.0
BH2012(pLGF14)	129.0
BH2012(pLGF5)	25.8
BH2012(pLGF17)	8.7

[a] Thioredoxin activity is expressed as nanomoles of NADPH oxidized per minute per milligram of extract protein. It was determined by monitoring insulin reduction. The reaction mixture contained 0.25 mg of bovine insulin, 0.6 μmol of EDTA, 10 μmol of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.6), 0.11 μmol of NADPH, and 0.2 μg of *E. coli* thioredoxin reductase in a final volume of 120 μl. The reaction mixture was incubated at 37°C for 20 min and stopped by adding 0.5 ml of 6 M guanidine hydrochloride–50 mM Tris (pH 8.0)–10 mM DTNB. The change in optical density was measured at 412 nm.

[b] TrxA$^+$ parent of BH2012.

were selected among transformants and analyzed for the presence of thioredoxin activity by the insulin precipitation assay. Five transformants contained significant thioredoxin activity and were further assayed by the DTNB reduction assay (Table 1). The five positive colonies were obtained from an *Hpa*II digestion and have a recombinant plasmid containing a common 1.02-kb *Hpa*III fragment (pLGF5, pLGF6, pLGF8, pLGF13, and pLGF14 [Fig. 1A]). One large

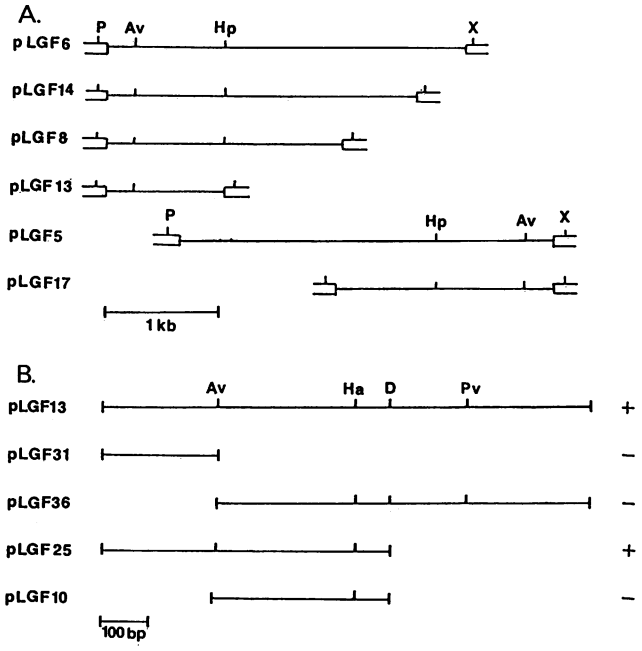

FIG. 1. (A) Structures of pUC13 derivatives containing the *Anabaena* thioredoxin gene. The open bars represent pUC13 vector DNA near the cloning site (*Acc*I). The inserts of plasmids pLGF6, pLGF14, and pLGF5 were found to contain additional *Hpa*II sites, but the locations of these were not determined. (B) Restriction maps of plasmid pLGF13 and subclones from pLGF13. A designation of + or − indicates ability of the plasmid to complement strain BH5262 growth on the minimal plate with glutathione. Restriction sites: P, *Pst*I; Av, *Ava*II; Hp, *Hpa*II; X, *Xba*I; Ha, *Hae*II; D, *Dde*I; Pv, *Pvu*I. kb, Kilobase.

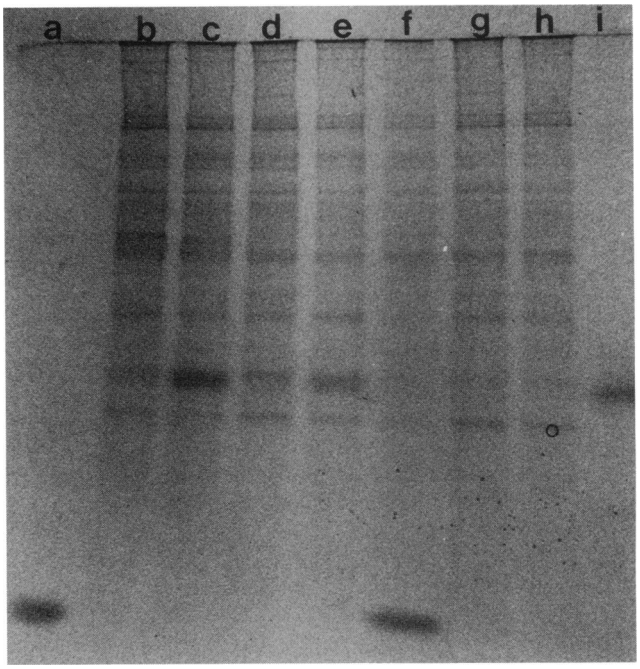

FIG. 2. Identification of *Anabaena* thioredoxin in *E. coli* extracts. Heat-treated extracts made from *E. coli* JF510 cultures carrying various plasmids were analyzed on a 15% polyacrylamide gel. Lanes: a, *E. coli* thioredoxin standard; b, pLGF13 without IPTG; c, pLGF13 with IPTG; d, pLGF14 without IPTG; e, pLGF14 with IPTG; f, pCJF4 with IPTG; g, pUC13 without IPTG; h, pUC13 with IPTG; i, *Anabaena* thioredoxin standard.

colony, which was obtained by transformation of the ligation mixture containing the entire *Anabaena* genomic DNA digested with *Hpa*II, contained significant thioredoxin activity (Table 1, pLGF17) and also a 1.02-kb *Hpa*II DNA fragment. Plasmid pLGF13 contains only a 1.02-kb *Hpa*II fragment insert, whereas the other plasmids contain additional *Anabaena* DNA fragments. The insert in pLGF13 contains an *Ava*II restriction site (Fig. 1A). The other plasmids that contain the 1.02-kb *Hpa*II fragment also have an *Ava*II restriction site toward one end of the insert. The location of the *Ava*II site in the 1.02-kb *Hpa*II fragment in plasmids pLGF5 and pLGF17 suggests an opposite orientation compared with the other plasmids (Fig. 1A). From the restriction digestion patterns of the cloned DNA fragment, the inserts in these plasmids did not appear to be originally contiguous in the genomic DNA and probably resulted from random ligation of *Hpa*II fragments.

Subcloning of a region containing the thioredoxin gene. Figure 1B shows the restriction map of the insert of pLGF13. To locate the *Anabaena* thioredoxin gene in the insert of pLGF13, subclones were constructed and strains containing the subclones were tested for complementation of the *E. coli trxA gshA* strain BH5262 (Fig. 1B). Plasmids pLGF13 and pLGF25 could complement this strain, whereas pLGF31 and pLGF36 did not. Plasmid pLGF25 contains the 650-bp *Hpa*II-*Dde*I fragment. These results indicated that the *Ava*II site is in the coding region of the *Anabaena* thioredoxin gene and that the two original plasmids, pLGF5 and pLGF17, must have their inserts in an orientation opposite to that of the *lac* promoter of pUC13. The thioredoxin gene in these plasmids appear to use an *Anabaena* promoter for expression.

FIG. 3. Nucleotide sequence of the thioredoxin gene from *Anabaena* sp. strain PCC 7119. The nucleotides are numbered 5′ to 3′ from the upstream *Hpa*II cleavage site. A possible ribosome-binding site, AGGT, is underlined. The region of inverted repeat is depicted by opposing arrows. The number above the sequence indicates the first base of each row. @, Termination codon.

To verify that the *Anabaena* gene which complements the *E. coli trxA* mutation encodes *Anabaena* thioredoxin, heat-treated crude extracts of strain BH2012 derivatives containing plasmids with either the *E. coli* or *Anabaena* thioredoxin gene were subjected to polyacrylamide gel electrophoresis. Figure 2 indicates that the insert of pLGF13 and pLGF14 encodes a protein that migrates to the same position as authentic *Anabaena* thioredoxin and that this protein was induced by addition of IPTG. These results indicated that expression of the *Anabaena* thioredoxin gene in pLGF13 and pLGF14 is under control of the *lac* promoter.

Sequence analysis of the *Anabaena* thioredox in gene. The DNA sequence of the 650-bp insert of pLGF25 was determined by the dideoxy chain termination method with [^{35}S]dATP. The nucleotide sequence determined from both strands and the corresponding deduced amino acid sequence are shown in Fig. 3. The protein encoded by this DNA minus the methionine is identical to the thioredoxin sequence previously reported (11).

The DNA sequence includes 147 bp of 5′ flanking region and 182 bp of 3′ flanking region of the coding sequence. A possible Shine-Dalgarno ribosome-binding site (12), AGGT, is located 8 nucleotides 5′ to the initiation codon. The sequence downstream from the coding region contains one stable stem-and-loop structure ($\Delta G = -16.9$ kcal/mol) (Fig. 3) followed by a T-rich region. This stem-and-loop structure could serve as a rho-independent transcription termination signal. When the coding DNA sequence was compared with that of the *E. coli* gene encoding thioredoxin, it showed 55% homology. As expected from a comparison at the protein level, major variations appeared at the coding regions for the N- and C-terminal amino acids. Approximately half of the homologous amino acids between *E. coli* and *Anabaena* thioredoxins are encoded by the same codons.

Purification of *Anabaena* thioredoxin from *E. coli*. To increase expression of the *Anabaena* thioredoxin gene in *E. coli*, we transferred the insert of pLGF13 to a plasmid very similar to the pUC series, except for a *tac* promoter rather than the *lac* promoter, to generate plasmid pFB30. *Anabaena* thioredoxin was purified from JF510 containing plasmid pFB30 after IPTG induction. The purification procedure of *Anabaena* thioredoxin from *E. coli* culture is described in Materials and Methods. The procedure previously reported for *Anabaena* thioredoxin (10) did not yield a homogeneous protein when used on *E. coli* extracts. To remove a low-molecular-weight contaminating protein, we used a chromatofocusing step in place of carboxymethyl Sepharose. Table 2 presents the results of purification of *Anabaena* thioredoxin from *E. coli* extracts. Thioredoxin

TABLE 2. Purification of *Anabaena* thioredoxin from *E. coli* JF510 cells carrying the *Anabaena* thioredoxin gene on a plasmid (pFB30)

Fraction	Total protein (mg)	Sp act[a]	Total activity (10^4 nmol/mg of protein)	% Recovery
Heat treated	2,740	5.17	1.417	
DEAE	372	31.3	1.160	82
G-50	21	262.6	0.543	38
PBE 94	3.1	946.1	0.293	21

[a] Specific activity is expressed as nanomoles of NADPH oxidized per minute per milligram of protein. The assay procedure is described in the legend to Table 1.

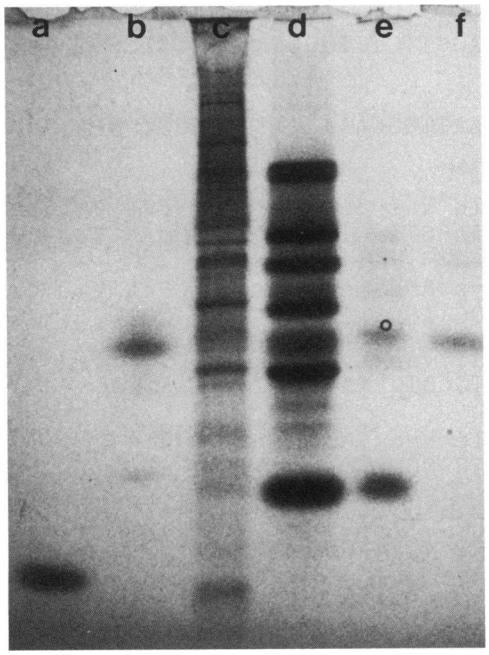

FIG. 4. Purification of *Anabaena* thioredoxin from *E. coli* cultures. Heat-treated extract was prepared from IPTG-induced JF510(pFB30) cells. The protein was purified from heat-treated extract by ammonium sulfate fractionation, DEAE-Sephacel column, Sephadex G-50 column, and PBE 94 chromatofocusing gel. Samples after each step were monitored on native polyacrylamide gels (15%). Lanes: a, *E. coli* thioredoxin; b, *Anabaena* thioredoxin; c, heat-treated fraction; d, DEAE fraction; e, G-50 fraction; f, PBE 94 fraction.

was purified 180-fold from heat-treated extracts after use of the chromatofocusing gel.

On native polyacrylamide gels, the thioredoxin coded on plasmid pFB30 exhibited the same mobility as authentic *Anabaena* thioredoxin (Fig. 4). On calibrated SDS gels, the molecular weight of the plasmid-coded thioredoxin was estimated to be 11,500 (data not shown). This is within experimental error of the known molecular weight of *Anabaena* thioredoxin (11,700 [10]).

To further verify that the plasmid-encoded protein was identical to *Anabaena* thioredoxin, we compared the kinetics of the reaction of this protein with those of authentic *Anabaena* thioredoxin. The K_m for *E. coli* thioredoxin reductase with the plasmid-encoded thioredoxin was very similar to that of authentic *Anabaena* thioredoxin but was

TABLE 3. Reduction of thioredoxins with *E. coli* thioredoxin reductase[a]

Thioredoxin from:	K_m (μM)	K_{cat} (min^{-1})[b]
E. coli	2.5	1,250
Anabaena sp. strain 7119	24	1,300
Anabaena sp. (coded on plasmid pFB30)	22	1,330

[a] Assay mixtures contained 100 mM Tris hydrochloride (pH 8.0), 2 mM EDTA, 0.1 mg of bovine serum albumin per ml, 0.5 mM DTNB, and 0.24 mM NADPH in a volume of 0.5 ml. Thioredoxins were added in the concentration range of 0.08 to 8.0 μM. The reaction was initiated by adding *E. coli* thioredoxin reductase at a concentration of 36 nM as determined from flavin A_{464}. The increase in A_{412} was monitored, and the activities were calculated by method 1 in reference 20.

[b] K_{cat}, Molecules of DTNB reduced per molecule of thioredoxin calculated from V_{max} of reaction.

TABLE 4. Phage growth on *E. coli* cells harboring cloned thioredoxin genes

Phage	Strain	Phage growth in the presence of[a]:		
		pUC13	pCJF4[b]	pLGF13(pLGF17)
T7	BH2012	−	+	−
T7 *tas*1	BH2012	−	+	+
M13mp2	JF510	−	+	+
f1	JF510	−	+	+[c]

[a] +, Growth; −, no growth.

[b] Plasmid pCJF4 is a pUC13 derivative carrying the *E. coli* thioredoxin gene (18).

[c] Wild-type f1 phage formed very tiny, unclear plaques on strain JF510(pLGF13) or JF510(pLGF17).

approximately 10-fold larger than when *E. coli* thioredoxin was used as a substrate (Table 3). The turnover numbers for all substrates were approximately equal. The lower binding of *Anabaena* thioredoxin to *E. coli* thioredoxin reductase has been attributed to structural differences between the algal and *E. coli* thioredoxins in the active-site region (8).

Biological characterization of *Anabaena* thioredoxin in *E. coli*. Thioredoxin-deficient mutants have several phenotypes that can be tested to determine whether *Anabaena* thioredoxin can replace *E. coli* thioredoxin in vivo. The original clones were selected for complementation of the reduced colony size of a $TrxA^-$ strain and, as shown in Fig. 1B, plasmid pLGF13 complements a *gsh trxA* mutant. Although strain BH2012 is unable to use methionine sulfoxide to satisfy its methionine requirement, strains BH2012(pLGF13) and BH2012(pLGF17) can use methionine sulfoxide as a methionine source, indicating that *Anabaena* thioredoxin serves as a cofactor for *E. coli* methionine sulfoxide reductase in vivo. The growth rate in minimal medium with methionine sulfoxide was the same for BH2012(pLGF13) and MC1061, the $trxA^+$ parent of BH2012 (data not shown). In contrast, strains BH2012(pLGF13) and BH2012(pLGF17) did not support growth of T7 phage, indicating that *Anabaena* thioredoxin is unable to serve as a subunit for the gene 5 protein to produce an active T7 DNA polymerase (Table 4). These strains contain more thioredoxin activity than a non-plasmid-containing $TrxA^+$ parental strain (Table 1). However, when high titers of T7 phage were plated on strain BH2012(pLGF13), mutant phage that could form plaques were obtained. T7 phage from 20 plaques were plaque purified. All 20 plated with equal efficiency on BH2012(pLGF13) and MC1061 ($trxA^+$ parent of BH2012) or BH2012(pCJF4) (plasmid containing *E. coli trxA* gene) but did not form plaques on BH2012. These mutant T7 phage, described T7 *tas* 1 to 20 (thioredoxin-altered specificity), presumably contain an altered gene 5 protein that can use *Anabaena* thioredoxin to reconstitute an active DNA polymerase. The mutant phage retained the requirement for a host-encoded thioredoxin and can use either *E. coli* or *Anabaena* thioredoxin. Strains JF510(pLGF13) and JF510(pLGF17) support the growth of filamentous phages M13mp2 and f1, although the plaques formed were smaller than on a strain with an *E. coli* thioredoxin (Table 4). These results indicated that *Anabaena* thioredoxin has sufficient structural homology to *E. coli* thioredoxin to serve in the unknown function required for filamentous phage assembly.

DISCUSSION

The structural gene for *Anabaena* sp. strain PCC 7119 thioredoxin was cloned in *E. coli* by size selection of

fragments identified by heterologous hybridization and complementation of an *E. coli trxA* mutant. Heterologous hybridization has been used to isolate several cyanobacterial genes such as the gene for glutamine synthetase (7) and two members of the 32-kilodalton thylakoid membrane protein gene family (5). In contrast, the phosphoenolpyruvate carboxylase gene (*ppc*) from *Anacystis nidulans* was cloned in *E. coli* by direct complementation of the *ppc* mutation (17).

Previous experiments on purified *Anabaena* thioredoxin indicated that it may serve as an analog of *E. coli* thioredoxin. It is a substrate for *E. coli* thioredoxin reductase and ribonucleotide reductase (10). As expected, derivatives of an *E. coli trxA* strain containing the *Anabaena* thioredoxin gene appear to be $TrxA^+$ for all phenotypes observed except for the growth of phage. Phage T7 was unable to form plaques, and phages M13 and f1 formed smaller plaques than on a $TrxA^+$ strain. It has been estimated that approximately 5% of the thioredoxin molecules in *E. coli* B cells are bound to the gene 5 protein to produce an active T7 polymerase (26, 27). From an analysis of heat-treated *E. coli* extracts, we estimated that the strains containing various pLGF plasmids produce two to three times as much thioredoxin activity as do wild-type cells (Table 1). Thus, it seems unlikely that *Anabaena* thioredoxin is not produced at levels sufficient to support T7 replication. Rather, these results imply that phage T7 DNA polymerase requires a specific amino acid sequence of thioredoxin to form an active complex. Although model-building studies show a similar conformation in the active centers of both *Anabaena* and *E. coli* thioredoxins (8), alterations in other parts of the protein may affect binding to T7 polymerase. Brändén and co-workers (3) have proposed that the region around amino acid residues 74 to 77 and 91 to 93 are critical for thioredoxin interaction with thioredoxin reductase and the gene 5 protein. For example, the *E. coli* mutant *tsnC7007* produces a thioredoxin with an aspartic acid substituted for the glycine at position 92. This mutant thioredoxin has a higher K_m with thioredoxin reductase and does not form an active T7 DNA polymerase (16). Although both *Anabaena* and *E. coli* thioredoxins show a high degree of homology in this region, *Anabaena* thioredoxin has a serine residue at position 74 in place of the glycine in *E. coli* thioredoxin (11). The *Anabaena* thioredoxin substitution is more subtle than that of the *E. coli tsnC7007* protein, but the results seem to be analogous. The *E. coli* thioredoxin reductase and *Anabaena* thioredoxin interaction showed a 10-fold higher K_m than the homologous reaction (Table 3), and the *Anabaena* thioredoxin did not form an active complex with the gene 5 protein in vitro (F. K. Gleason and A. Holmgren, unpublished data). However, phage T7 was able to mutate to use the *Anabaena* thioredoxin. This mutation presumably changes the gene 5 protein structure so that an active polymerase is formed with *Anabaena* thioredoxin. The assembly of filamentous phages does not appear to have equally stringent requirements for thioredoxin structure, and these are able to function, although not as efficiently, with the heterologous protein.

The amino acid sequences of at least five different thioredoxins have been compared and found to be homologous, suggesting that they might have evolved from the same ancestral gene. This is supported by our finding that there is 55% homology in the coding sequences of the *E. coli* and *Anabaena* thioredoxin genes. This compares to 50% homology at the amino acid level. It was found that codon use in other *Anabaena* genes such as *rbcA* (4), *nifH* (25), and *nifK* (24) displayed many asymmetries. In contrast, codons used in the *Anabaena* thioredoxin gene do not reflect this asymmetry, especially for Leu, Val, and Ala. The *Anabaena* thioredoxin gene uses about 44% of nonoptimal codons based on the codon usage of *E. coli*, whereas the *E. coli* thioredoxin gene uses only 23% nonoptimal codons. This indicates that the classification of optimal or nonoptimal codons in *Anabaena* may be different from that of *E. coli* or that the *Anabaena* thioredoxin gene might not be highly expressed. Use of 44% nonoptimal codons may result in difficulties in achieving high-level expression of *Anabaena* thioredoxin in *E. coli*.

Plasmids pLGF13 and pLGF17, which have their transcription units in reverse orientation of the *lac* promoter of vector DNA, also contained significant thioredoxin activities, indicating that the promoter of the *Anabaena* thioredoxin gene may function in *E. coli* cells. At position 120 of the sequence in Fig. 3, the sequence TATAAT corresponds to an *E. coli* −10 sequence, and the TTCACG sequence at 101 corresponds to an *E. coli* −35 sequence. This region may serve as a functional promoter in *E. coli* and perhaps in *Anabaena* sp. under certain conditions (31). At positions 31 and 48, the sequences CATAAA and CTT TGCA are very similar to the *Anabaena nif* promoters reported by Tumer et al. (31).

A comparison of Tables 1 and 2 suggests that an insert of the *Anabaena* thioredoxin gene in a pUC vector derivative containing the *tac* promoter results in significantly decreased expression compared with a pUC vector containing the *lac* promoter. However, large-scale purification of a strain containing pLGF13 produced similar results (data not shown). This suggests that strains containing the high-copy-number vector with an *Anabaena* thioredoxin gene may be unstable. Similar results have been found for a pUC13 vector containing *E. coli* thioredoxin (unpublished data). Attempts to alter growth conditions to yield a stable strain are in progress and should allow large-scale production of *Anabaena* thioredoxin.

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LITERATURE CITED

1. **Ashton, A. R., T. Brennan, and L. E. Anderson.** 1980. Thioredoxin-like activity of thylakoid membranes. Thioredoxin catalyzing the reductive inactivation of glucose-6-phosphate dehydrogenase occurs in both soluble and membrane-bound form. Plant Physiol. **66**:605–608.
2. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein to dye bindings. Anal. Biochem. **72**:248–254.
3. **Brändén, C.-I., H. Eklund, and B.-O. Söderberg.** 1983. Structure and functional similarities in thioredoxins from *Escherichia coli* and bacteriophage T4, p. 223–230. *In* A. Larsson, S. Orrenius, A. Holmgren, and B. Mannervik (ed.), Functions of glutathione. Raven Press, New York.
4. **Curtis, S. E., and R. Haselkorn.** 1983. Isolation and sequence of the gene for the large subunit of ribulose-1,5-bisphosphatase carboxylase from the cyanobacterium *Anabaena* 7120. Proc. Natl. Acad. Sci. USA **80**:1835–1839.
5. **Curtis, S. E., and R. Haselkorn.** 1984. Isolation, sequence, and expression of two members of the 32Kd thylakoid membrane protein gene family from the cyanobacterium *Anabaena* 7120. Plant Mol. Biol. **3**:249–258.
6. **Davis, B. J.** 1964. Disc electrophoresis—II. Method and appli-

cation to human serum proteins. Ann. N.Y. Acad. Sci. **121:** 404–427.
7. **Fisher, R., R. Tuli, and R. Haselkorn.** 1981. A cloned cyanobacterial gene for glutamine synthetase functions in *Escherichia coli*, but the enzyme is not adenylated. Proc. Natl. Acad. Sci. USA **78:**3393–3397.
8. **Gleason, F. K.** 1986. Thioredoxin in the cyanobacteria: structure and functional homology to thioredoxins from other organisms, p. 21–30. *In* A. Holmgren, C.-I. Brändén, H. Jörnvall, and B.-M. Sjöberg (ed.), Thioredoxin and glutaredoxin systems: structure and function. Raven Press, New York.
9. **Gleason, F. K., and T. D. Frick.** 1980. Adenosylcobalamin-dependent ribonucleotide reductase from the blue-green alga, *Anabaena* sp. J. Biol. Chem. **225:**7728–7733.
10. **Gleason, F. K., and A. Holmgren.** 1981. Isolation and characterization of thioredoxin from the cyanobacterium, *Anabaena* sp. J. Biol. Chem. **256:**8306–8309.
11. **Gleason, F. K., M. M. Whittaker, A. Holmgren, and H. Jörnvall.** 1985. The primary structure of thioredoxin from the filamentous cyanobacterium *Anabaena* sp. 7119. J. Biol. Chem. **260:** 9567–9573.
12. **Gold, L., D. Pribnow, T. Schneider, S. Shinedling, and B. S. Singer.** 1981. Translational initiation in prokaryotes. Annu. Rev. Microbiol. **35:**365–403.
13. **Heidecker, G., J. Messing, and B. Gronenborn.** 1980. A versatile primer for DNA sequencing in the M13mp2 cloning system. Gene **10:**69–73.
14. **Holmgren, A.** 1979. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J. Biol. Chem. **254:**9627–9632.
15. **Holmgren, A.** 1985. Thioredoxin. Annu. Rev. Biochem. **54:** 237–271.
16. **Holmgren, A., G.-B. Kallis, and B. Nordström.** 1981. A mutant thioredoxin from *Escherichia coli tsnC7007* that is nonfunctional as subunit of phage T7 DNA polymerase. J. Biol. Chem. **256:**3118–3124.
17. **Kodaki, T., F. Katagiri, M. Asano, K. Izui, and H. Katsuki.** 1985. Cloning of phosphoenolpyruvate carboxylase gene from a cyanobacterium, *Anacystis nidulans*, in *Escherichia coli*. J. Biochem. **97:**533–539.
18. **Lim, C.-J., D. Geraghty, and J. A. Fuchs.** 1985. Cloning and nucleotide sequence of the *trxA* gene of *Escherichia coli* K-12. J. Bacteriol. **163:**311–316.
19. **Lim, C.-J., B. Haller, and J. A. Fuchs.** 1985. Thioredoxin is the bacterial protein encoded by *fip* that is required for filamentous bacteriophage f1 assembly. J. Bacteriol. **161:**799–802.
20. **Luthman, M., and A. Holmgren.** 1982. Rat liver thioredoxin and thioredoxin reductase: purification and characterization. Biochemistry **21:**6628–6633.
21. **Maeda, K., A. Tsugita, D. Dalzoppo, F. Vilbois, and P. Schurmann.** 1986. Further characterization and amino acid sequence of m-type thioredoxins from spinach chloroplasts. Eur. J. Biochem. **154:**197–203.
22. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53:**154–162.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 382–389. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Mazur, B. J., and C.-F. Chui.** 1982. Sequence of the gene coding for the β-subunit of dinitrogenase from the blue-green alga *Anabaena*. Proc. Natl. Acad. Sci. USA **79:**6782–6786.
25. **Mevarech, M., D. Rice, and R. Haselkorn.** 1980. Nucleotide sequence of a cyanobacterial *nifH* gene coding for nitrogenase reductase. Proc. Natl. Acad. Sci. USA **77:**6476–6480.
26. **Modrich, P., and C. C. Richardson.** 1975. Bacteriophage T7 deoxyribonucleic acid replication *in vitro*. Bacteriophage T7 DNA polymerase: an enzyme composed of phage- and host-specified mutants. J. Biol. Chem. **250:**5515–5522.
27. **Nordström, B., H. Randahl, I. Slaby, and A. Holmgren.** 1981. Characterization of bacteriophage T7 DNA polymerase purified to homogeneity by antithioredoxin immunoadsorbent chromatography. J. Biol. Chem. **256:**3112–3117.
28. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101–106.
29. **Russell, M., and P. Model.** 1984. Characterization of the cloned *fip* gene and its product. J. Bacteriol. **157:**526–532.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
31. **Tumer, N. E., S. J. Robinson, and R. Haselkorn.** 1983. Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen. Nature (London) **306:**337–342.
32. **Weber, K., J. R. Pringle, and M. Osborn.** 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. Methods Enzymol. **26:**3–27.
33. **Wolosiuk, R. A., and B. B. Buchanan.** 1977. Thioredoxin and glutathione regulate photosynthesis in chloroplasts. Nature (London) **266:**565–567.