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Isolation, Sequence, and Expression in *Escherichia coli* of an Unusual Thioredoxin Gene from the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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Two sequences with homology to a thioredoxin oligonucleotide probe were detected by Southern blot analysis of *Anabaena* sp. strain PCC 7120 genomic DNA. One of the sequences was shown to code for a protein with 37% amino acid identity to thioredoxins from *Escherichia coli* and *Anabaena* sp. strain PCC 7119. This is in contrast to the usual 50% homology observed among most procaryotic thioredoxins. One gene was identified in a library and was subcloned into a pUC vector and used to transform *E. coli* strains lacking functional thioredoxin. The *Anabaena* strain 7120 thioredoxin gene did not complement the *trxA* mutation in *E. coli*. Transformed cells were not able to use methionine sulfoxide as a methionine source or support replication of T7 bacteriophage or the filamentous viruses M13 and f1. Sequence analysis of a 720-base-pair *Taq*I fragment indicated an open reading frame of 115 amino acids. The *Anabaena* strain 7120 thioredoxin gene was expressed in *E. coli*, and the protein was purified by assaying for protein disulfide reductase activity, using insulin as a substrate. The *Anabaena* strain 7120 thioredoxin exhibited the properties of a conventional thioredoxin. It is a small heat-stable redox protein and an efficient protein disulfide reductase. It is not a substrate for *E. coli* thioredoxin reductase. Chemically reduced *Anabaena* strain 7120 thioredoxin was able to serve as reducing agent for both *E. coli* and *Anabaena* strain 7119 ribonucleotide reductases, although with less efficiency than the homologous counterparts. The *Anabaena* strain 7120 thioredoxin cross-reacted with polyclonal antibodies to *Anabaena* strain 7119 thioredoxin. However, this unusual thioredoxin was not detected in extracts of *Anabaena* strain 7120, and its physiological function is unknown.

Thioredoxin is a small (M_r, approximately 12,000) heat-stable redox protein found in cells of a wide variety of both procaryotes and eucaryotes. The active site is a cystine disulfide with the amino acid sequence --Trp-Cys-Gly-Pro-Cys--. The reduced form was originally described in yeast extracts, in which it acts as a reducing agent for sulfate reductase (2). This protein was subsequently shown to be a hydrogen donor for *Escherichia coli* ribonucleotide reductase (23), a function which it can also serve in other cells. Thioredoxin has been reported as a cofactor, presumably as reducing agent, for methionine sulfoxide reductase in *E. coli* (10) and adenosine 3′-P 5′-phosphosulfate transferase (39). It can function as a protein disulfide reductase and has been shown to effectively reduce the disulfides in proteins such as insulin (17) and NADP-dependent malate dehydrogenase (35). The latter activity of thioredoxin may enable it to regulate in vivo enzyme activity via redox control.

The dithiol form of thioredoxin is involved in bacteriophage reproduction in *E. coli*. The T7 gene 5 protein (DNA polymerase) forms a complex with the host thioredoxin (32), and this complex increases the processivity of the polymerase (37). *E. coli* mutants lacking thioredoxin will not support T7 replication. The filamentous viruses M13 and f1 also require *E. coli* thioredoxin for coat protein assembly (29, 33). In these interactions, the reduced conformation of thioredoxin is required, but not redox activity per se (20).

E. coli cells contain only one type of thioredoxin; however, multiple thioredoxins have been reported to occur in the bacterium *Corynebacterium nephridii* (25) and in spinach chloroplasts (7). Thioredoxins in chloroplasts can regulate the enzymes of carbon dioxide metabolism such as fructose bisphosphatase (43) and glucose-6-phosphate dehydrogenase (3), by reducing or oxidizing sulfhydryl groups of these enzymes (8). Two thioredoxins were also reported to occur in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7119 (44). Subsequent purification and characterization of both thioredoxins led to the conclusion that only one of these proteins was actually a thioredoxin, i.e., a small redox protein which functioned as a protein disulfide reductase (13, 42). On isolation and characterization of the gene for *Anabaena* strain 7119 thioredoxin, using an *E. coli* probe, only one thioredoxin fraction was found (27). However, using a synthetic oligonucleotide probe, two putative thioredoxin genes were identified in the related organism, *Anabaena* sp. strain PCC 7120. One of the thioredoxin genes from *Anabaena* strain 7120 was isolated and sequenced. This *Anabaena* strain 7120 thioredoxin exhibits only a 37% amino acid sequence homology to the thioredoxin from *Anabaena* strain 7119. We report here the purification and characterization of the unusual *Anabaena* strain 7120 thioredoxin from extracts of *E. coli* cells.

MATERIALS AND METHODS

Materials. All restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, Klenow fragment of DNA polymerase I, bacterial alkaline phosphatase, and isopropyl-β-D-thiogalactoside were purchased from Bethesda Research

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Laboratories, Inc., Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Indianapolis, Ind. All enzymes were used as specified by the manufacturer. Dithiothreitol (DTT), 5,5′-dithiobis(1-nitrobenzoic acid), NADPH, bovine insulin, molecular weight standards, acrylamide, and anti-rabbit immunoglobulin alkaline phosphatase conjugate were from Sigma Chemical Co., St. Louis, Mo. DEAE-Sephacel, Sephadex G-50, and the Mono Q column were purchased from Pharmacia, Inc., Piscataway, N.J. YM-5 ultrafiltration membranes (molecular weight cutoff, 5,000) were obtained from the Amicon Corp., Lexington, Mass. [γ-^{32}P]ATP (approximately 7,000 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. [α-^{32}P]dATP (approximately 800 Ci/mmol), [5-^{3}H]CDP, and [5-^{3}H]CTP were purchased from Dupont, NEN Research Products, Boston, Mass. Unlabeled nucleotides were obtained from P-L–Pharmacia, Milwaukee, Wis. All oligonucleotides were synthesized on controlled-pore glass supports in an automated DNA synthesizer (model 380A; Applied Biosystems, Foster City, Calif.) and purified as reported previously (1). *E. coli* thioredoxin and *Anabaena* strain 7119 thioredoxin were purified from *E. coli* strains containing plasmid-encoded thioredoxins by previously published procedures (19, 27). *E. coli* thioredoxin reductase and rat liver thioredoxin reductase were purified on 2′,5′-ADP–Sepharose as described by Luthman and Holmgren (30). A homogeneous preparation of *E. coli* ribonucleotide reductase was a generous gift of B.-M. Sjöberg. *Anabaena* strain 7119 ribonucleotide reductase was purified as described previously (11).

Bacterial strains and phages. A thioredoxin-deficient (*trxA*) strain, BH2012, which is *E. coli* K-12 F^{-} *araD139*? *galU galK hsr rpsL metA46 argHl trxA7004 ilvC*::Tn*5*, was used as a recipient for transformation. As previously reported (26), *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 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^{-} *araD139*? *galU galK hsr rpsL argHl 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 either *gshA*$^{+}$ or *trxA*$^{+}$ do not require glutathione for growth (B. Haller and J. Fuchs, unpublished results). The *trxA7004* derivatives of strains 71.18 (15), JF510 (*trxA7004 ilvC*::Tn*5*), and JF517 (JF510 *recA srl-300*::Tn*10*) were constructed by phage P1 transduction and used to test the ability of filamentous bacteriophages M13 and fl to form plaques. Phages M13mp2, obtained from J. Messing, and wild-type fl, obtained from M. Russel, were used for tests of phage replication.

Southern blot analysis. DNA from *Anabaena* sp. strain PCC 7120 was prepared as described previously (9). Chromosomal DNA was digested with restriction endonucleases, subjected to agarose gel electrophoresis, and transferred to a nitrocellulose filter (31). The oligonucleotide (25 pmol) was end labeled with [γ-^{32}P]ATP (40 pmol; approximately 7,000 Ci/mmole) by T4 polynucleotide kinase to a specific activity of 10^{9} cpm/μg. Excess label was removed from the oligonucleotide by passage through a Sep-Pak cartridge (Waters Associates, Inc., Milford, Mass.). The labeled probe (10^{7} cpm) was hybridized to the nitrocellulose filter in a mixture of 6× SSC (1× SSC is 0.15M NaCl plus 0.015 M sodium citrate, pH 7.0), 0.5% sodium dodecyl sulfate (SDS), and 5× Denhardt solution (31) at 37°C for 16 h in a volume of 10 ml. The filter was subsequently washed with two changes (30 min each) of 6× SSC–0.5% SDS at 37°C and two changes (30 min each) of 1× SSC–0.5% SDS at 37°C.

Screening of genomic library. An *Anabaena* sp. strain PCC 7120 genomic library in lambda vector L47.1 was a kind gift from Jim Golden. The recombinant bacteriophage library was screened by plaque hybridization (4), using a synthetic oligonucleotide as the hybridization probe. The hybridization conditions were identical to those described above for Southern blot analysis, except that the filters were incubated in a total volume of 100 ml with a probe concentration of 2 × 10^{6} cpm/ml. Posthybridization treatments were equivalent to those described above.

DNA sequence determination. DNA sequence was determined by the dideoxynucleotide chain termination method (34), using M13 single-stranded templates and site-directed oligonucleotide primers.

Enzyme assays. Thioredoxin-catalyzed reduction of insulin by DTT was monitored as a turbidity increase at 650 nm (17) in a Hewlett-Packard 8450A split-beam spectrophotometer (Hewlett-Packard Co., Palo Alto, Calif.). Homogeneous thioredoxins from *Anabaena* strain 7119 or *E. coli* were used as standards. Reaction mixtures contained 80 mM potassium phosphate buffer (pH 7.0), 0.8 mM EDTA, 1 mg of bovine insulin, and 1 mM DTT in a final volume of 1 ml. The reaction was initiated by the addition of thioredoxin to the reaction curvette.

Thioredoxin activity was also determined by monitoring insulin reduction in the presence of rat liver thioredoxin reductase and NADPH (method 4 in reference 30). Reaction mixtures contained 10 μmol of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6), 0.25 mg of bovine insulin, 0.6 μmol of EDTA, 0.11 μmol of NADPH, and 0.5 μg of partially purified rat liver 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 in 50 mM Tris (pH 8.0) containing 10 mM 5,5′-dithiobis(1-nitrobenzoic acid). The change in A_{412} was measured.

E. coli 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 to 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 polyethyleneimine-cellulose plates (Brinkmann Instruments, Inc., Westbury, N.Y.) as described previously (12). Labeled deoxynucleotide was eluted from the plate with 0.7 M $MgCl_2$ and added to PCS scintillation cocktail (Amersham Corp., Arlington Heights, Ill.). Radioactivity was determined in a Searle Mark IV scintillation spectrometer. The activity of *Anabaena* ribonucleotide reductase was determined by measuring the reduction of [^{3}H]CTP. The reaction mixture contained 65 mM HEPES (pH 8.0), 8 mM $CaCl_2$, 0.004 mM dATP, 1.3 mM [^{3}H]CTP (approximately 4,000 cpm/nmol), 0.8 mM DTT, and 0.008 mM adenosylcobalamin in a final volume of 0.060 ml. The reaction was initiated by addition of *Anabaena* ribonucleotide reductase and incubated at 35°C in the dark. The reaction was stopped by boiling the reaction tubes, and

deoxyribonucleotides were separated from the mixture as described above.

Purification of *Anabaena* strain 7120 thioredoxin. *E. coli* cells harboring the *Anabaena* strain 7120 thioredoxin gene on plasmid pAn673.2 were grown on enriched medium to an optical density at 660 nm of 0.2, and isopropyl-β-D-thiogalactoside was added to 0.1 mM final concentration. Growth was continued to late stationary phase. Cells were harvested by centrifugation and stored at −20°C. Approximately 40 g (wet weight) of cells was suspended in buffer containing 50 mM Tris hydrochloride (pH 7.5) and 1 mM EDTA, and cells were disrupted by sonication. Unbroken cells and debris were removed by centrifugation at 10,000 × *g* for 20 min. The supernatant was applied to a DEAE-Sephacel column (10 by 2.5 cm) which was preequilibrated with buffer containing 20 mM Tris hydrochloride (pH 7.5), and 1 mM EDTA. Thioredoxin activity, as determined by the insulin precipitation assay, washed through the column. The active fractions were pooled and concentrated by ultrafiltration, using a YM-5 membrane at 70 lb/in^2 of N_2.

The DEAE pool was applied to a column of Sephadex G-50 (90 by 1.6 cm) and eluted with 50 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. Active fractions were pooled and concentrated as above. The thioredoxin was approximately 80% pure at this stage as determined by native polyacrylamide gel electrophoresis run at neutral pH (14). Minor impurities were removed by fast protein liquid chromatography on a Mono Q column. The column was preequilibrated with 0.05 M ethanolamine hydrochloride buffer (pH 9.0) containing 1 mM EDTA. Protein was eluted from the column with a gradient of NaCl, 0 to 0.3 M, in the same buffer.

Anabaena thioredoxin was estimated from A_{280} and A_{310} as for *E. coli* thioredoxin (22). Purity of the fractions was determined by native polyacrylamide gel electrophoresis. Neutral discontinuous gels containing 15% acrylamide were run in Tris hydrochloride buffer (pH 7.0) and stained with Coomassie blue (14). The molecular weight of *Anabaena* strain 7120 thioredoxin was determined by SDS-polyacrylamide gel electrophoresis in 15% acrylamide with standard proteins as described by Weber et al. (41).

Amino acid analysis. Denatured thioredoxin was prepared by dissolving 0.46 mg (77 μM) of protein in 0.5 ml of buffer containing 0.4 M Tris hydrochloride (pH 8.1), 2 mM EDTA, and 6 M guanidine hydrochloride. The aromatic amino acid content was estimated from the second-derivative UV spectra in a Hewlett-Packard model 8450A spectrophotometer (24). The sample was then reduced by addition of 5 μl of 0.5 M DTT, flushed with N_2, and carboxymethylated with iodoacetate (13). Amino acid analyses were performed on a Beckman model 119 analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

Fluorescence measurements. Fluorescence spectra were recorded in a Perkin-Elmer MPF-44A spectrometer (The Perkin-Elmer Corp., Norwalk, Conn.). Measurements were made in a 1-cm-path-length cell in a total volume of 1 ml. The excitation wavelength was 280 nm, and emission was recorded from 290 to 400 nm as described previously (28).

Reaction with antibody to *Anabaena* strain 7119 thioredoxin. Polyclonal antibodies to *Anabaena* strain 7119 thioredoxin were raised in a rabbit as described previously (28). The antibodies were precipitated from serum with 40% saturated ammonium sulfate and dialyzed against Tris-buffered saline (150 mM NaCl). This preparation was used for immunoblotting without further purification.

The basic procedure for detecting thioredoxins by immunoblotting was adapted from that described by Tsang et al. (40). Reactive bands were visualized by alkaline phosphatase staining (5).

RESULTS

Identification and isolation of a gene for thioredoxin in *Anabaena* strain 7120. The primary structure of thioredoxin(s) from *Anabaena* sp. strain 7120 is not known; however, the sequence at the active site of this protein is highly conserved even between procaryotic and eucaryotic organisms (18). Based on the amino acid sequence at the active site of *Anabaena cylindrica* thioredoxin, --F-W-A-P-W-C-G-P-C-T-M-V-- (21), and the codon usage bias in *Anabaena* strain 7120, the following oligonucleotide, complementary to the mRNA was synthesized: 5′-ACCATGGTGCAAGGA CCGCACCAAGGAGCCCAGAA-3′.

In Southern blot analysis, this oligonucleotide hybridizes to two *Eco*RI and *Hin*dIII restriction fragments of total *Anabaena* strain 7120 DNA (Fig. 1). This result suggests that *Anabaena* strain 7120 contains at least two genes for thioredoxin.

The oligonucleotide was used as a hybridization probe to screen an estimated 5 × 10^3 recombinant lambda phage, representing approximately 40 genome equivalents of *Anabaena* strain 7120 DNA. After three sequential screenings, two clones exhibiting positive hybridization signals were isolated and one of these clones, λAn670, was selected for further examination. Southern blot analysis of DNA from λAn670 identified a 4.4-kilobase-pair (kbp) *Hin*dIII/*Eco*RV restriction fragment that contains sequences complementary to the oligonucleotide probe. This fragment was cloned into the vector pUC9 to generate the recombinant plasmid pAn672. Subclones of this fragment were generated by digestion with *Taq*I endonuclease, and the products were cloned into the *Acc*I site of M13mp19. A unique clone, pAn673 (and its complement), containing a 720-bp *Taq*I fragment was identified by plaque hybridization.

Sequence analysis of the thioredoxin gene. The nucleotide sequence of both strands of the insert in pAn673 was determined by using chemically synthesized oligonucleotide primers. The 720-bp fragment contains a single open reading frame corresponding to a 115-amino-acid polypeptide with significant sequence identity to thioredoxins from *E. coli* and *Anabaena* strain 7119 (See below). The nucleotide sequence of the noncoding strand of the *Anabaena* strain 7120 thioredoxin gene is displayed in Fig. 2 with the open reading frame indicated by the juxtaposed translation. Two methionine residues are found near the amino terminus, either of which could serve as the site of translation initiation. The sequence of the oligonucleotide probe is positioned directly above the corresponding sequence of the thioredoxin gene in Fig. 2. These sequences are only complementary at 27 of 35 nucleotide residues.

The isolated thioredoxin gene is located on a 23-kbp *Eco*RI fragment and on a 9.5-kbp *Hin*dIII restriction fragment of *Anabaena* strain 7120 genomic DNA (data not shown, but see Fig. 1). The second of the original recombinant lambda isolates was characterized and shown to contain the same thioredoxin gene.

Characterization of the *Anabaena* strain 7120 thioredoxin gene in *E. coli*. The double-stranded replicative form of pAn673, containing the 720-bp fragment, was digested in the multiple-cloning-site region with the restriction endonucleases *Pst*I and *Bam*HI. The purified insert was transferred into the appropriately digested vectors pUC8 and pUC9 to

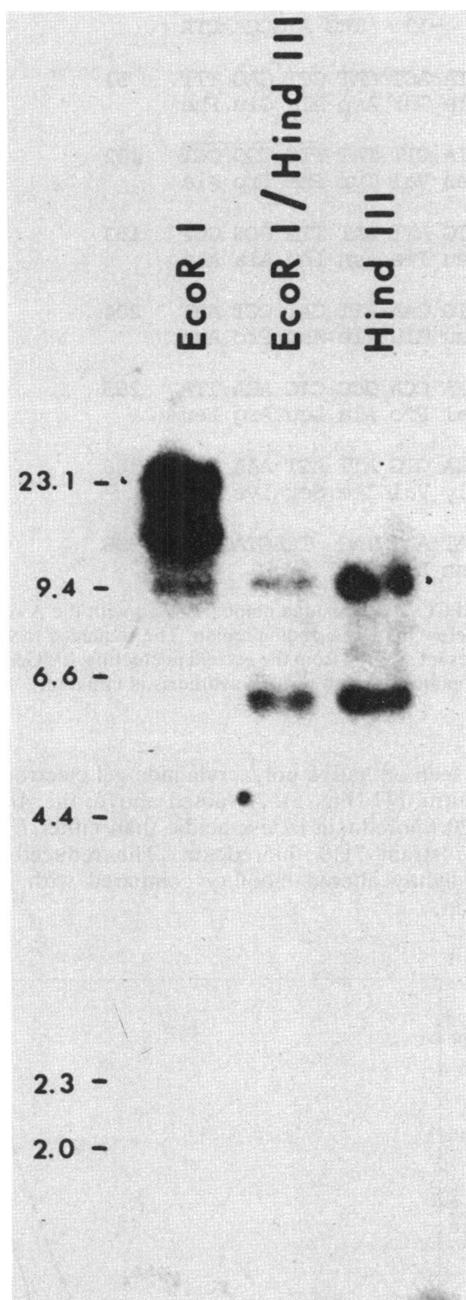

FIG. 1. Identification of *Anabaena* DNA fragments containing genes for thioredoxin. Total *Anabaena* DNA was digested with the endonuclease *Eco*RI or *Hin*dIII or a mixture of both enzymes, and fragments were separated by electrophoresis, blotted, and hybridized with labeled oligonucleotide. Fragments of *Hin*dIII-digested lambda DNA were used as molecular-size standards.

yield recombinant plasmids pAn673.1 and pAn673.2, respectively. In pAn673.2, the thioredoxin gene is positioned in-frame with the *lac* open reading frame, and a fusion protein of thioredoxin with a 15-amino-acid extension at the N terminus could be made if the *lac* ribosome-binding site and start codon were used. However, the *Anabaena* thioredoxin gene could also use its own ribosome-binding site and start reading at the Met codon at position 13 (Fig. 2). In either case, the *lac* promoter could be utilized. Plasmid 673.1 contains the *Anabaena* strain 7120 thioredoxin gene in the opposite orientation and was used as a control. To verify that the *Anabaena* thioredoxin gene could be expressed in *E. coli* without the additional amino acids at the N terminus, the 720-bp insert of pAn673.2 was transferred into pT7-5 and pT7-6 via the *Eco*RI and *Hin*dIII sites (38). These plasmids were transferred into strain JF521 containing plasmid pGP1-2. After heat induction to accumulate T7 RNA polymerase, rifampin was added to inhibit *E. coli* RNA polymerase and the cells were pulsed with [^{14}C]methionine. Cell extracts were run on SDS-polyacrylamide gels. Subsequent autoradiographs showed predominant production of a protein with a molecular weight of 12,000 (data not shown, but see Fig. 6). Since the larger chimeric protein was not detected in this system, the data indicate that the *Anabaena* strain 7120 thioredoxin is synthesized from its own ribosome-binding site and ATG start codon rather than being made as a fusion protein.

Plasmids pAn672, pAn673.1, and pAn673.2 were transformed into *E. coli trxA* mutants and tested for complementarity. *E. coli trxA* mutant strains exhibit reduced colony size when plated on enriched medium. It had previously been shown that the large colony size was restored in transformants containing plasmids which code for *E. coli* or *Anabaena* strain 7119 thioredoxin (27). Plasmids pAn672, pAn673.1, and pAn673.2 were unable to restore large colony size in any of the strains tested. None of the plasmids containing the *Anabaena* strain 7120 thioredoxin gene was able to complement the *gshA trxA* double mutant BH5262, which requires glutathione for growth, nor were any of the plasmids able to restore the wild-type phenotype of strain BH2012, which is unable to use methionine sulfoxide to satisfy its methionine requirement.

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 (32). A strain containing plasmid pAn673.2 does not support T7 growth (Table 1). This is also true for the strain producing *Anabaena* strain 7119 thioredoxin. However, when high titers of T7 were plated on strain BH2012 containing plasmid pLGF13, which codes for *Anabaena* strain 7119 thioredoxin, mutant phage which will replicate in this strain could be isolated. An isolate of this phage has been designated T7tas1 for thioredoxin-altered specificity and presumably has a mutation in the T7 gene 5 (27). The T7tas1 mutant polymerase will also accept other foreign thioredoxins (28). However, strains containing the *Anabaena* strain 7120 thioredoxin will not support the growth of even the less demanding virus. Also, unlike strains containing *Anabaena* strain 7119 thioredoxin, those producing *Anabaena* strain 7120 thioredoxin cannot support the filamentous phages M13 and f1. These viruses apparently require the host thioredoxin for coat protein assembly.

Isolation and characterization of *Anabaena* strain 7120 thioredoxin from *E. coli*. Unlike thioredoxin from *Anabaena* strain 7119, the protein encoded by the *Anabaena* strain 7120 gene was not reduced by *E. coli* thioredoxin reductase, but it was reduced by the less specific rat liver thioredoxin reductase. Activity could be detected in crude extracts of *E. coli* strains containing the *Anabaena* strain 7120 thioredoxin at levels of 3 to 12 nmol of NADPH oxidized/min per mg of extract protein. Unfortunately, insufficient rat liver thioredoxin reductase was available to use this procedure for complete isolation of the *Anabaena* strain 7120 thioredoxin. *Anabaena* strain 7120 thioredoxin could also be detected in extracts of *E. coli* by its ability to catalyze the reduction of

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                                                             -13   TCG ATGCCAACTA

ATG GAG ACT GCA ATG AGT AAG GGT GTA ATC ACC ATA ACT GAT GCT GAG TTT      51
Met Glu Thr Ala Met Ser Lys Gly Val Ile Thr Ile Thr Asp Ala Glu Phe

GAA AGC GAA GTG TTG AAA GCC GAG CAG CCT GTA TTA GTT TAT TTC TGG GCT     102
Glu Ser Glu Val Leu Lys Ala Glu Gln Pro Val Leu Val Tyr Phe Trp Ala

TCT TGG TGC GGG CCG TGC CAG TTG ATG TCA CCA CTG ATT AAT TTA GCG GCT     153
Ser Trp Cys Gly Pro Cys Gln Leu Met Ser Pro Leu Ile Asn Leu Ala Ala

AAT ACC TAC AGC GAT CGC TTG AAA GTC GTC AAA CTG GAA ATT GAT CCT AAT     204
Asn Thr Tyr Ser Asp Arg Leu Lys Val Val Lys Leu Glu Ile Asp Pro Asn

CCC ACA ACT GTC AAA AAA TAC AAG GTA GAA GGC GTA CCA GCC CTC AGA TTA     255
Pro Thr Thr Val Lys Lys Tyr Lys Val Glu Gly Val Pro Ala Leu Arg Leu

GTA AAA GGA GAA CAG ATA TTA GAC TCT ACC GAA GGA GTG ATT AGT AAA GAT     306
Val Lys Gly Glu Gln Ile Leu Asp Ser Thr Glu Gly Val Ile Ser Lys Asp

AAA TTA CTC AGT TTC CTA GAT ACG CAT TTA AAT AAT AAT TAG  TCAGTAGTCA    358
Lys Leu Leu Ser Phe Leu Asp Thr His Leu Asn Asn Asn End
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FIG. 2. Nucleotide sequence of the thioredoxin gene from *Anabaena* sp. strain PCC 7120. Residue numbers begin with the A of the first methionine codon. The derived amino acid sequence for thioredoxin is displayed below the DNA-coding region. The sequence to which the oligonucleotide probe hybridizes is overlined. A possible ribosome-binding site is present starting from the second nucleotide, TGGAG, which occurs 6 nucleotides upstream from the second Met codon. This is apparently the point at which protein synthesis is initiated.

insulin in the presence of DTT. Purified *Anabaena* strain 7120 thioredoxin was as effective as *E. coli* thioredoxin in this reaction (Fig. 3). Approximately 5 μg of either thioredoxin caused a precipitation reaction to occur in about 15 min. Insulin reduction with DTT alone occurs in 40 min under these conditions. This assay was used to monitor the thioredoxin activity during purification.

Anabaena strain 7120 thioredoxin could also be detected in *E. coli* extracts by immunoblotting with polyclonal antibodies to *Anabaena* strain 7119 thioredoxin. Cross-reaction with 7120 thioredoxin is less intense than with *Anabaena* strain 7119 thioredoxin, even after purification (Fig. 4). This antibody preparation does not react as intensely with *E. coli* thioredoxin. A fivefold-greater amount of *E. coli* thioredoxin does result in a minor reaction band (lane 4, Fig. 4). The blots indicate that the *Anabaena* strain 7120 thioredoxin is slightly less acidic at pH 7.5 than *Anabaena* strain 7119 thioredoxin. This property may explain its lack of retention on the DEAE-Sephacel column.

After fast protein liquid chromatography on a Mono Q column, *Anabaena* strain 7120 thioredoxin was homogeneous as seen on native polyacrylamide gel electrophoresis run at neutral pH (Fig. 5). As noted above, the *Anabaena* strain 7120 thioredoxin is less acidic than either *E. coli* or *Anabaena* strain 7119 thioredoxin. The reduced protein exhibits slightly altered mobility compared with oxidized thioredoxin.

TABLE 1. Plaque formation of phage on *E. coli* cells containing cloned thioredoxin genes

Phage	Plaque formation with given thioredoxin type (plasmid)[a]			
	None (pUC13)	*E. coli* (pCJF4)	*Anabaena* strain 7119 (pLGF13)	*Anabaena* strain 7120 (pAn673.2)
T7	−	+	−	−
T7tas1[b]	−	+	+	−
M13mp2	−	+	+	−
f1	−	+	+[c]	−

[a] +, Plaque formation; −, no plaque formation.

[b] Mutant-T7-designated, thioredoxin-altered sensitivity (27).

[c] Wild-type f1 phage formed very small unclear plaques on strains containing plasmid pLGF13.

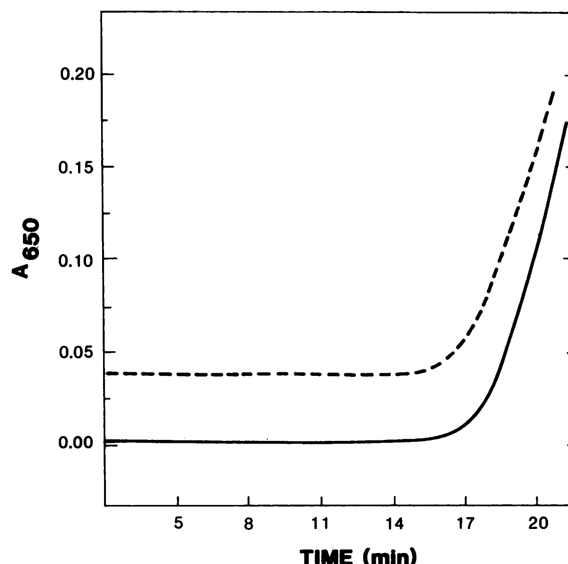

FIG. 3. Thioredoxin-catalyzed reduction of insulin. Reaction mixtures contained 80 mM potassium phosphate buffer (pH 7.0), 0.8 mM EDTA, 1 mg of bovine insulin, and 1 mM DTT in a final volume of 1 ml. The reaction was initiated by addition of thioredoxin, and the increase in turbidity was monitored at 650 nm and 25°C. The blank cuvette contained the same components minus thioredoxin. Symbols: ——, 5 μg of *E. coli* thioredoxin added to the cuvette; – – – –, 4.5 μg of *Anabaena* strain 7120 thioredoxin added to the cuvette.

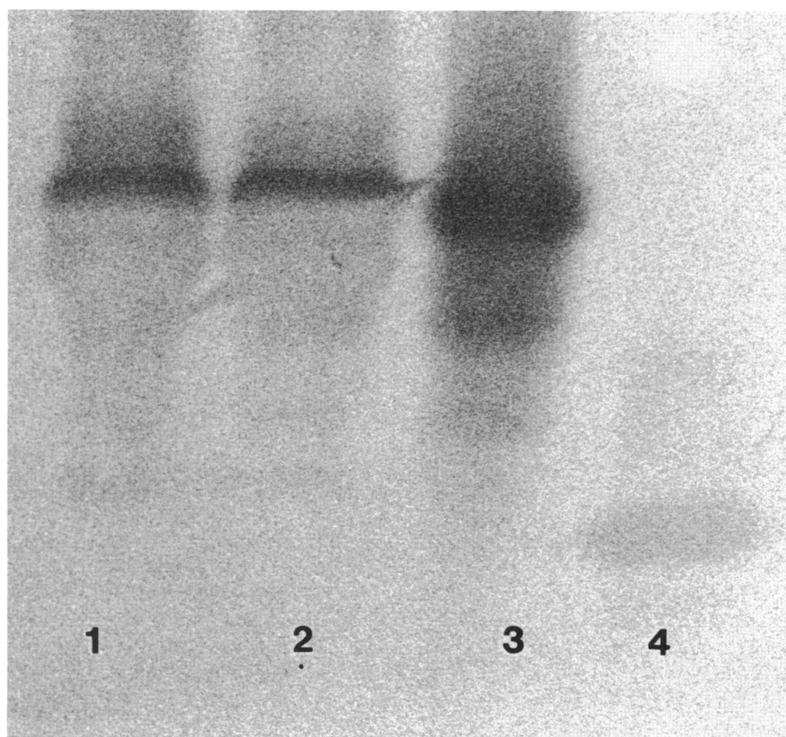

FIG. 4. Western immunoblot analysis of thioredoxins. Proteins were separated by electrophoresis on native 15% polyacrylamide gels. The proteins were then transferred to nitrocellulose paper and reacted with antibodies to *Anabaena* strain 7119 thioredoxin. Reactive conjugates were visualized by alkaline phosphatase staining. Lane 1, Heat-treated extract of *E. coli* containing plasmid pAn673.2 (approximately 20 μg of total protein); lane 2, 1 μg of purified *Anabaena* strain 7120 thioredoxin; lane 3, 1 μg of purified *Anabaena* strain 7119 thioredoxin; lane 4, 5 μg of purified *E. coli* thioredoxin.

The molecular weight of *Anabaena* strain 7120 thioredoxin was estimated by chromatography on Sephadex G-50. The algal protein elutes at the same position as *E. coli* thioredoxin, indicating a relative molecular weight of approximately 12,000 (data not shown). *Anabaena* strain 7120 thioredoxin also exhibits the same mobility on SDS-polyacrylamide gel electrophoresis as *Anabaena* strain 7119 thioredoxin (Fig. 6). The amino acid composition of *Anabaena* strain 7120 thioredoxin after hydrolysis is shown in Table 2. This agrees within experimental error with the composition predicted from the gene sequence if translation starts at the second Met codon, using the cyanobacterial

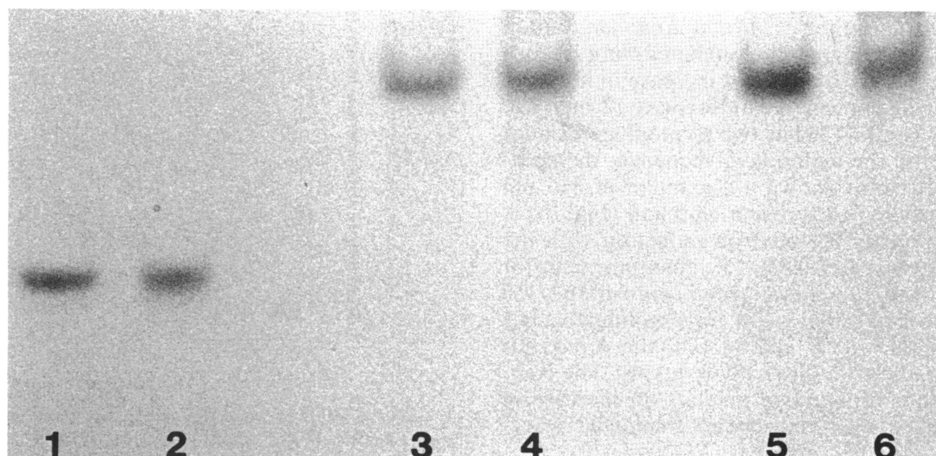

FIG. 5. Separation of thioredoxins by native polyacrylamide gel electrophoresis. Approximately 5 μg of each protein was loaded onto a 15% polyacrylamide gel. Thioredoxins were reduced with 20 mM DTT (final concentration). Lane 1, *E. coli* thioredoxin, oxidized; lane 2, *E. coli* thioredoxin, reduced; lane 3, *Anabaena* strain 7119 thioredoxin, oxidized; lane 4, *Anabaena* strain 7119 thioredoxin, reduced; lane 5, *Anabaena* strain 7120 thioredoxin, purified by fast protein liquid chromatography on a Mono Q column and oxidized; lane 6, *Anabaena* strain 7120 thioredoxin, reduced.

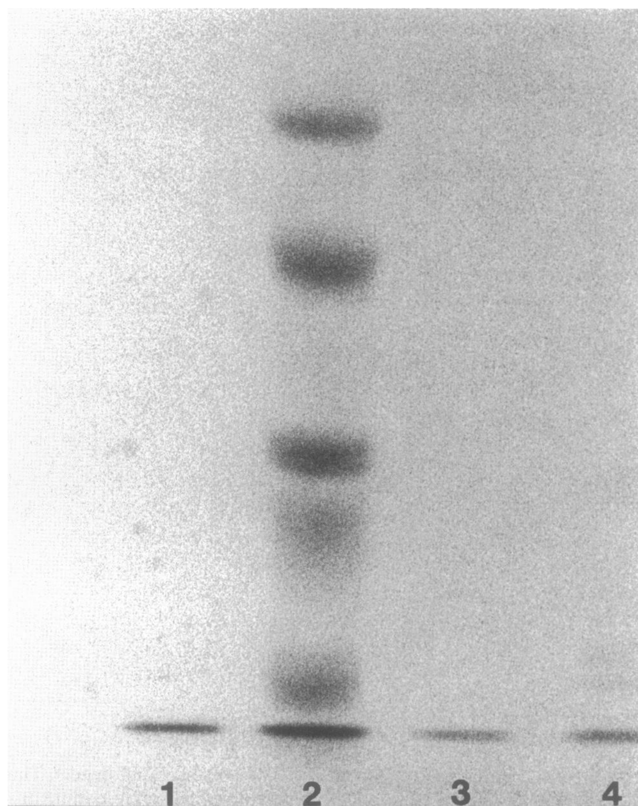

FIG. 6. Molecular weight determination of *Anabaena* strain 7120 thioredoxin on SDS-polyacrylamide gel electrophoresis. Approximately 2 μg of protein was run on 15% SDS gels. Lane 1, *Anabaena* strain 7120 thioredoxin; lane 2, molecular weight markers (top to bottom: egg albumin, 45,000; pepsin, 34,700; trypsinogen, 24,000; beta-lactoglobulin, 18,400; lysozyme, 14,000; *E. coli* thioredoxin, 11,700); lane 3, thioredoxin from *Anabaena* strain 7119; lane 4, thioredoxin from *Anacystis nidulans* R2.

ribosome-binding site (Fig. 2). From the biochemical data, it appears that the thioredoxin is indeed synthesized with these signals.

Fluorescence spectra. Both *E. coli* and *Anabaena* strain 7119 thioredoxins have tryptophan-dominated fluorescence spectra which show a two- to threefold increase in intensity on reduction (data not shown; see references 12 and 16). Although *Anabaena* strain 7120 has two tryptophan residues in the same position in the amino acid sequence, the spectrum of the oxidized form shows a maximum at 310 nm which has been attributed to tyrosine emission (Fig. 7). A slight increase in intensity is noted on reduction with the appearance of a maximum at 340 nm. The quantum yields for oxidized, reduced, and denatured *Anabaena* strain 7120 thioredoxin are shown in Table 3 with corresponding values for *Anabaena* strain 7119 and *E. coli* thioredoxin. A red shift on unfolding is characteristic of all thioredoxins. The *Anabaena* strain 7120 thioredoxin also exhibits an increase in quantum yield at the longer tryptophan-dominated wavelength.

Reaction with ribonucleotide reductases. Presumably *Anabaena* strain 7120 thioredoxin does not restore the wild-type phenotype in *E. coli trxA* mutants because of its lack of cross-reaction with *E. coli* thioredoxin reductase. However, the cyanobacterial thioredoxin is a moderately good reducing agent for *E. coli* ribonucleotide reductase (Table 4). Changes occur mainly in the binding constant in the heterologous reaction. *Anabaena* strain 7120 thioredoxin also serves as reducing agent for the coenzyme B12-dependent reductase from *Anabaena* strain 7119. As with the *E. coli* enzyme, reduced catalytic efficiency compared with the other thioredoxins is noted (Table 5).

TABLE 2. Amino acid composition of *Anabaena* strain 7120 thioredoxin[a]

Amino acid	From gene sequence	Found on analysis
Ala	6	7–8
Cys	2	2[b]
Asx	12	12
Glx	11	12–13
Phe	3	3
Gly	5	5–6
His	1	1
Ile	6	6
Lys	10	10
Leu	14	14
Met	1	1
Pro	6	6
Arg	2	3
Ser	8	8
Thr	7	7
Val	11	11
Trp	2	2[c]
Tyr	3	3[c]

[a] Estimated molecular weight, 12,201.
[b] As carboxymethylated cysteine.
[c] From UV analysis.

DISCUSSION

Southern analysis of genomic DNA from *Anabaena* strain 7120 suggests the presence of at least two thioredoxin genes. The derived amino acid sequence of the gene product which

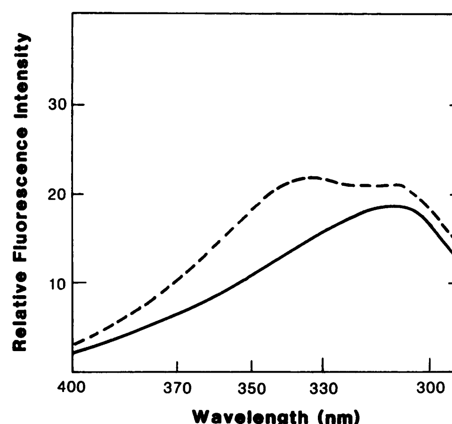

FIG. 7. Fluorescence emission spectra of *Anabaena* strain 7120 thioredoxin. Thioredoxin (A_{280}, 0.05) was dissolved in 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. Spectra were recorded after excitation at 280 nm. Temperature was maintained at 30°C. All buffers were degassed before use. The spectra were not corrected for monochromator and detector responses. Symbols: ——, oxidized thioredoxin; – – – –, reduced thioredoxin (1 mM DTT).

TABLE 3. Fluorescence properties of thioredoxins[a]

Thioredoxin	Quantum yield (10^2)[b]		
	Oxidized	Reduced	Denatured
E. coli	14 (345)	43 (345)	40 (360)
Anabaena strain 7119	19 (340)	36 (345)	45 (360)
Anabaena strain 7120	18 (310)	19 (310)	18 (315)
	14 (330)	21 (340)	35 (345)

[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. Thioredoxins were denatured by incubation for 15 min in 6 M guanidine hydrochloride in the above buffer. Solvent blanks were run and subtracted from the same spectra. The spectra were not corrected for monochromator and detector responses.

[b] Numbers in parentheses indicate the wavelength of maximum emission. Quantum yields were calculated on the basis of the Q of tryptophan in water = 0.144 (16).

was chosen for further analysis is shown in Fig. 8 in comparison with the primary structures of thioredoxins from *E. coli* and *Anabaena* strain 7119. Most bacterial thioredoxins exhibit approximately 50% amino acid identity when aligned from the active-site cystine disulfide. On comparison of the sequence of *Anabaena* strain 7119 thioredoxin with the partially characterized N-terminal sequence from *A. cylindrica* (21), 80% identity between these proteins is found. In contrast, the thioredoxin from *Anabaena* strain 7120 described here shows only 37% amino acid identity with that from the other cyanobacteria, although several altered residues can be considered conservative changes. The unusual *Anabaena* strain 7120 protein has the characteristic thioredoxin active-site sequence and a molecular weight of approximately 12,000. In addition, the *Anabaena* strain 7120 thioredoxin has some conserved residues which appear to be common to all bacterial thioredoxins. For example, a proline residue is always found at position 76 preceded by a hydrophobic residue (*E. coli* thioredoxin sequence numbering is used). The *Anabaena* strain 7120 thioredoxin also preserves the hydrophobic core of Phe-12, Phe-27, Tyr-70, and Val-81, substituted for Phe-81. A hydrophobic series of residues from 91 to 94, which is believed to be important in thioredoxin-protein interactions (6), is also partially conserved. A major change occurs at position 91, where *Anabaena* strain 7120 thioredoxin has a glutamic acid instead of a hydrophobic amino acid. This alteration and other chemically radical changes such as the Glu at 73 substituted for the positively charged Arg may account for the lack of cross-reactivity between *Anabaena* strain 7120 thioredoxin and various *E. coli* proteins and the inability of this thioredoxin to complement the *trxA* mutants. Thus, this *Anabaena* strain 7120 thioredoxin is not a substrate for *E. coli* thioredoxin reductase, although it is reduced by the less specific mammalian thioredoxin reductase. *Anabaena* strain 7120 thioredoxin will not support the replication of any

TABLE 4. Thioredoxins as reducing agents for *E. coli* ribonucleotide reductase[a]

Thioredoxin	K_m (μM)	kcat/min	Catalytic efficiency (kcat/K_m)
E. coli	2.0	130	65
Anabaena strain 7119	2.0	130	65
Anabaena strain 7120	22.0	173	8

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

TABLE 5. Thioredoxins as reducing agents for *Anabaena* ribonucleotide reductase

Thioredoxin	K_m (μM)	kcat/min	Catalytic efficiency (kcat/K_m)
E. coli	2.2	35	16
Anabaena strain 7119	2.0	36	18
Anabaena strain 7120	12.5	31	2.5

[a] CTP reductase activity was determined with dATP as effector and in the presence of 0.8 mM DTT as described in Materials and Methods.

coliphage, including the mutant T7tas1 strain which will accept a variety of heterologous thioredoxins. Lack of phage complementation may be due to protein structural changes or it may reflect the fact that *Anabaena* strain 7120 thioredoxin cannot be reduced in vivo. The inability of *E. coli* thioredoxin-minus mutants containing plasmid pAn673.2 to utilize methionine sulfoxide or to grow in the absence of reduced glutathione may also be due to the inability of *Anabaena* strain 7120 thioredoxin to be reduced in *E. coli* cells.

Although *Anabaena* strain 7120 thioredoxin does not function in *E. coli*, it is still a good protein disulfide reductase. It will catalyze the DTT-dependent reduction of insulin as efficiently as *E. coli* thioredoxin (Fig. 3). Chemically reduced *Anabaena* strain 7120 thioredoxin will also serve as reducing agent for *E. coli* ribonucleotide reductase (Table 4), although it is not as efficient as *Anabaena* strain 7119 thioredoxin in the heterologous reaction. The change is mainly in the binding constant, which suggests that three-dimensional structural differences in *Anabaena* strain 7120

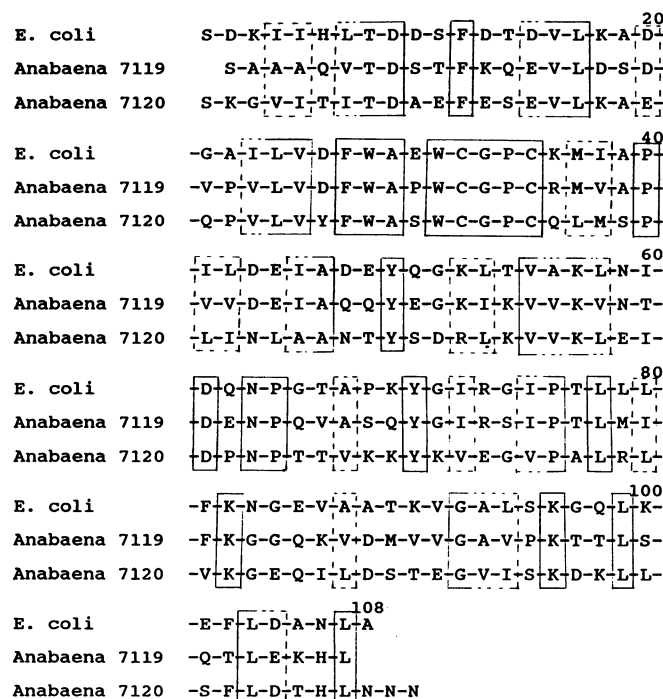

FIG. 8. Comparison of thioredoxin primary structures. Sequences were aligned from the active-site disulfide, residues 32 to 35. Numbering is based on the *E. coli* sequence. Identical residues are enclosed in solid lines. Conservative residue changes are enclosed in dashed lines.

thioredoxin make it less likely to bind to the reductase in this electron transfer reaction. A similar phenomenon is seen in the reaction with the more closely related *Anabaena* strain 7119 ribonucleotide reductase (Table 5). The reduced catalytic efficiency suggests that participation in ribonucleotide reduction is not the main in vivo function of *Anabaena* strain 7120 thioredoxin.

In contrast, *Anabaena* strain 7120 thioredoxin will cross-react with antibodies to *Anabaena* strain 7119 thioredoxin. It has previously been shown that antigenic sites are mainly in the region which lies C terminal to the thioredoxin active site (28). The cross-reaction of *Anabaena* strain 7120 thioredoxin presumably reflects an antigenic site with conserved primary and tertiary structures between the thioredoxins. The fluorescence data (Table 3 and Fig. 7) also indicate some structural similarity to other thioredoxins in the active-site region. Thioredoxin fluorescence is mainly due to Trp-28, which is buried behind the active-site disulfide bridge. The fluorescence of this residue is quenched in the oxidized form, and there is generally a two- to threefold increase in intensity on reduction. This increase is observed in *Anabaena* strain 7120 thioredoxin, although it is not as great as in other thioredoxins. The Trp-28 fluorescence is also more quenched in the oxidized form of *Anabaena* strain 7120 thioredoxin, which suggests that this phenomenon is not due solely to the proximity of the disulfide as has been proposed (16). In addition, a strong tyrosine emission is seen at 310 nm in the *Anabaena* strain 7120 thioredoxin, which is also characteristic of oxidized *E. coli* thioredoxin. Unlike *E. coli* thioredoxin, this tyrosine emission is still visible in the reduced form of the cyanobacterial protein. This is most likely due to the relatively quenched tryptophan emission and the rigid structure of thioredoxin which does not allow for efficient energy transfer (16). Also, unlike other thioredoxins, the *Anabaena* strain 7120 protein has an additional tyrosine at position 26 in place of a buried aspartic acid residue (Fig. 8) which will further contribute to the tyrosine emission intensity.

The cyanobacteria may be similar to spinach chloroplasts with two thioredoxin fractions, Tm and Tf, having different functions (7). However, the *Anabaena* strain 7120 thioredoxin shows no similarity to the partially sequenced spinach Tf (36) except in the active-site region. It is possible that two thioredoxins also exist in *Anabaena* sp. strain 7119, but the unusual fraction was overlooked. Cross-reaction with *E. coli* thioredoxin reductase is generally used to detect and purify the cyanobacterial thioredoxins. In addition, the unusual *Anabaena* strain 7120 thioredoxin does not adhere to anion-exchange columns which are used in thioredoxin purification (12). Only one thioredoxin gene was observed on Southern blot analysis of *Anabaena* strain 7119 genomic DNA (27). However, since the *E. coli* thioredoxin gene was used as a probe, there may not be sufficient sequence similarity to permit detection of a highly divergent cyanobacterial gene. Indeed, the isolated *Anabaena* strain 7120 thioredoxin gene does not hybridize to the second *Anabaena* strain 7120 gene in a Southern blot analysis of genomic DNA even under conditions of low stringency. The second *Anabaena* strain 7120 genomic sequence detected by the oligonucleotide probe may correspond to the more common procaryotic thioredoxin.

At present, there is no known function of the unusual *Anabaena* strain 7120 thioredoxin. Since it is an efficient protein disulfide reductase, it could play a regulatory role in cyanobacteria similar to that proposed for chloroplast thioredoxins. Western blot (immunoblot) analysis of *Anabaena* strain 7120 extracts showed the presence of only one thioredoxin, which suggests that only one of the proteins is expressed at detectable levels under laboratory growth conditions. It may be that expression of the unusual *Anabaena* strain 7120 thioredoxin occurs only in connection with specific processes such as heterocyst differentiation and nitrogen fixation.

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