Isolation and Characterization of Thioredoxin from the Cyanobacterium, Anabaena sp.*

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Thioredoxin from Anabaena sp. has been purified 800-fold with an assay based on the reduction of insulin disulfides by NADPH and the heterologous calf thymus thioredoxin reductase. The final material was homogeneous on polyacrylamide gel electrophoresis and had a molecular weight of 12,000; the NH2-terminal residue was serine and the COOH-terminal was leucine. Anabaena thioredoxin-(SH)2 is a hydrogen donor for the adenosylcobalamin-dependent Anabaena ribonucleotide reductase and is equally active with the iron-containing ribonucleotide reductase from Escherichia coli. Anabaena thioredoxin-S2 is a good substrate for E. coli thioredoxin reductase. We have compared the structure of Anabaena and E. coli thioredoxins. Clear structural differences between the proteins, compatible with the large evolutionary distance between the organisms, were seen with respect to total amino acid composition, isoelectric point, tryptic peptide maps, and a low immunological cross-reactivity. However, both thioredoxins contain a single oxidation-reduction active disulfide bridge with the amino acid sequence: Cy3-Gly-Pro-Cys-Lys. The tryptophan fluorescence emission of Anabaena thioredoxin-S2 increases more than 3-fold on reduction to thioredoxin-(SH)2. This behavior is identical with that of E. coli thioredoxin, suggesting a very similar overall folding of homologous molecules.

Thioredoxin is a small (Mr = 12,000) protein, containing an active center cystine disulfide/dithiol in its oxidized and reduced form, respectively. Initially, thioredoxin and the specific NADPH-dependent enzyme thioredoxin reductase were purified from E. coli as a hydrogen donor system for ribonucleotide reductase and thus believed to be required chiefly for DNA synthesis (1). Thioredoxin has since been studied in a variety of organisms and shown to function in other thiol-dependent oxidation-reduction reactions (for review, see Ref. 2).

Although thioredoxin presumably occurs in all living organisms, it has been purified to homogeneity and characterized in only a few. Existing data on thioredoxins from E. coli, yeast, and mammalian sources indicate considerable homology (2). A comparative study of thioredoxin from various species may help in understanding its molecular mechanism and also offer additional data on proteins and evolution comparable to that provided by cytochrome c (3).

Other than the protein from E. coli, no bacterial thioredoxin has been isolated and described as to structure and reactivity. In this paper we report the purification and some of the properties of thioredoxin from the filamentous cyanobacterium Anabaena 7119. The ribonucleotide reductase from this organism has recently been purified and shown to be an adenosylcobalamin-dependent reductase (4) comparable to that found in Lactobacillus leichmannii and other prokaryotes (5). This is in marked contrast to the iron-containing reductase found in E. coli and mammals (6). The data here reported show that the cyanobacterial thioredoxin is homologous to the protein from E. coli despite the large evolutionary distance between these organisms.

EXPERIMENTAL PROCEDURES AND RESULTS†

This paper describes the isolation of a homogeneous thioredoxin from Anabaena sp. and some of its characteristics. Thioredoxins have also been studied from E. coli (1), Lactobacillus leichmannii (26), the green alga Scenedesmus obliquus (27), yeast (24), and mammalian liver (25, 28). All these thioredoxins have certain properties in common. They are heat-stable proteins with molecular weights of approximately 12,000. In the reduced form they serve as hydrogen donors for E. coli as well as their homologous ribonucleotide reductases (6). This indicates identical thiol oxidation-reduction mechanisms and thus the same active center structure. On the other hand, the thioredoxins differ in their reactivity with thioredoxin reductases or antibodies, consistent with marked primary structural differences. A summary of these points is given in Table III.

Only thioredoxin from E. coli has been extensively characterized. Results from an x-ray crystallographic investigation to 2.8Å resolution demonstrate that this molecule represents a novel type of enzyme structure (29); the active center disulfide is located at the COOH-terminal end of a β-pleated sheet protruding out into the solution. Furthermore, the molecule consists of two prominent folding domains of secondary structure...
structure connected by a short hinge region. The folding of Anabaena thioredoxin-S₂ must be very similar to that of E. coli thioredoxin-S₂, since both share reactivity properties and are substrates for the otherwise highly specific thioredoxin reductase of E. coli (2). The structure of Anabaena thioredoxin may be represented as shown in Fig. 7. The amino acid sequence of the active center pentapeptide cleaved by trypsin and chymotrypsin digestion is identical in E. coli, Anabaena, and also in yeast thioredoxins (31). Fluorescence spectra of Anabaena and E. coli thioredoxin-S₂ and thioredoxin-(SH)₂ are a strong argument for placing the tryptophans in Anabaena in the same relative position as in E. coli (Trp-28 and Trp-31) (20). The low quantum yield of tryptophan fluorescence in thioredoxin-S₂ is attributed to quenching by the disulfide (21). The large increase in fluorescence on reduction is caused by Trp-28, which moves as the result of a localized conformational change (31).

Despite the identities in overall folding and active center residues, including conservation of thioredoxin reductase binding site(s), the peptide maps and immunological data show that Anabaena and E. coli thioredoxins have quite different primary structures. This is consistent with evolution of homologous proteins. From an evolutionary point of view, the cyanobacteria are considered to be direct descendants of an ancient group of microorganisms (32). The occurrence of an adenosylcobalamin-dependent ribonucleotide reductase in the majority of common cyanobacteria further confirms their ancient lineage (33). The fact that there exists a high degree of homology between the thioredoxin systems of phototrophic prokaryotes such as Anabaena and the geneologically distant E. coli (34) indicates that natural selection has conserved the thioredoxins throughout evolution, although the ribonucleotide reductases have diverged widely.

The role of thioredoxin in deoxyribonucleotide synthesis is presently uncertain since the discovery of the glutaredoxin system in E. coli (35) and the characterization of thioredoxin-negative mutants (36). However, thioredoxin is known to have other functions. Thioredoxin has been shown to act as a cofactor for the 3'-phosphateadenosine 5'-phosphosulfate sulfotransferase system of yeast (37) and cyanobacteria (38). As a regulatory factor for photosynthetic enzymes, three thioredoxin fractions have been purified from spinach (39). Two of these, from chloroplasts, function in specific regulation of enzymes of carbon dioxide fixation, fructose bisphosphatase and NADP-malate dehydrogenase. Both enzymes show increased activity after reduction by the chloroplast thioredoxin (39). Their ability to serve as hydrogen donor for E. coli ribonucleotide reductase, however, is minimal (40). Since the cyanobacteria are believed to be the evolutionary prototypes for chloroplasts (41, 42), the function of the Anabaena thioredoxin system described here in regulation of other aspects of cyanobacterial metabolism merits further investigation.

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Thioredoxin from Anabaena

**Supplemental Material to**

**Isolation and Characterization of Thioredoxin from the Cyanobacterium, Anabaena sp.*

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**Experimental Procedures**

Materials. Dithiothreitol, 5,5'-dithiobis-2-nitrobenzilic acid, NADPH, CMH, adenosine 5'-triphosphate and unlabeled nucleotides were purchased from Sigma Chemical Co., St. Louis, Mo. Thioredoxin (17) was from the Cyanobacterium Anabaena sp. (CM 52) was from Whatman, Ltd., England. Ammonium sequence reagents were as in 7. Cyanobacterium thioredoxin was prepared by the procedure described in reference 18. E. coli thioredoxin was prepared as described (7). E. coli ribonucleotide reductase and E. coli glutaredoxin were prepared from E. coli K 12 according to published methods (15, 19, 20). E. coli glutaredoxin and thioredoxin (7) and calf thymus thioredoxin (16) have been previously described.

Growth of the cyanobacterium. Anabaena sp. 7119 (AXCC 2515) was a gift of A. Neilan (Institute for Marine and Cold Water Research, Stockholm, Sweden). Cultures were routinely maintained on liquid medium, CG-50 (13) at room temperature and constant illumination. Large quantities of cells were grown on the same medium previously described (18).

**Enzyme Assays.** Thioredoxin activity was routinely determined by monitoring the oxidation of NADPH in the presence of insulin and 24 μM (54 nM units) (100), of thioredoxin from calf thymus (100) as described in equation 1:

1. Thioredoxin-S NADPH thoredoxin reductase, thioredoxin (7), NADP

2. Thioredoxin-S NADPH insulin insulin (insulin)

Purified Anabaena thioredoxin serves as hydroxon donor for E. coli ribonucleotide reductase, while the homologous adenosylcobalamin-dependent reductase from Anabaena Activity was determined by measuring the conversion of 1-μM [6,7-3H]NADPH to the corresponding deoxyribonucleotides. The assay procedure for E. coli thioredoxin reductase is described in reference 12 and for the heterologous reductases in 4. Separation of nucleotides was characterized by two-dimensional chromatography on polyvinylidene fluoride plates (13).

The activity of Anabaena thioredoxin was used as substrate for E. coli thioredoxin reductase was determined in the presence of NADPH and 7.5-μM [3H]thioredoxin (18). The reduction of NADPH was determined by monitoring the increase in absorbance at 412 nm (14).

**Protein determination.** Protein concentration in crude extracts and partially purified fractions was estimated from the ratio of absorbance at 280 nm to 260 nm (15). The concentration of pure thioredoxin from Anabaena and E. coli was determined with a molar absorptivity of 11,700 M⁻¹ cm⁻¹ at 280 nm (16). The value for Anabaena thioredoxin was also determined by amino acid analysis.

**Coomassie blue.** Protein standards (10 to 150 μg) were prepared and run at 250 nm in 1% Tri-glycine, pH 9.8. The protein bands were stained with commassie brilliant blue in methanol-acetic acid-5% H₂O (4:1-9:5:100) by volume and destained with the same solvent. E. coli thioredoxin-S and glutaredoxin 173 were used as markers.

**Amino Acid Analysis.** Salt-free aliquots of lyophilized Anabaena thioredoxin (5 μg) were hydrolyzed with 0.35 N of 6 N HCl containing 0.1% phenol at 1100 for 24 h. After hydrolysis the amino acid composition and methionine content was determined after performic acid oxidation (16).

**SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis was performed by the technique of Laemmli (1). Molecular weight markers were from Pharmacia, Sweden. The Coomassie blue stained gel was photographed, and the molecular weight of the protein bands were determined by comparing with the molecular weight of the markers. The protein concentration of the sample was estimated by monitoring the absorbance at 280 nm (18).

**COOM-assay procedure.** The COOM assay was utilized for the thioredoxin reductase activity. The concentration of thioredoxins was determined by monitoring the ADP formed. A molar absorptivity of 6,850 M⁻¹ cm⁻¹ at 340 nm was used (19).

**Results**

**Purification of Anabaena thioredoxin from 40 g of Cells**

| Fraction | Total protein (μg) | Total activity (μg) | Specific activity (nmol/min) |
|----------|-------------------|--------------------|------------------------------|
| 1. Crude extract | 19,500 | 380 | 0.023 |
| 2. Centrifuged | 19,500 | 516 | 0.027 |
| 3. DEAE pool | 24 | 76 | 3.2 |
| 4. DEAE pool | 6.9 | 42 | 6.2 |
| 5. CM-cellulose pool | 1.7 | 30 | 17.7 |

**Purification of thioredoxin.** Material from fraction 6 was homogenous on native polyacrylamide gel electrophoresis (Fig. 1) and at pH 8.6. Anabaena thioredoxin-S had mobility similar to calf thymus thioredoxin (17) demonstrating a higher isoelectric point (over pH 4.5) (22).

**Fig. 1. Native polyacrylamide gel electrophoresis.** Thioredoxin (1.5 μg) was separated by electrophoresis in a 5% gel containing 0.1% SDS. (A) Lane 1, E. coli thioredoxin; lane 2, E. coli thioredoxin-S; lane 3, A. aureus thioredoxin; lane 4, A. increta thioredoxin; lane 5, A. variabilis thioredoxin. (B) Lane 1, A. variabilis thioredoxin; lane 2, A. variabilis thioredoxin; lane 3, A. variabilis thioredoxin. The values are the same as those previously published for E. coli thioredoxin (17).

**Fig. 2.** Ribonucleotide triphosphate reductase reaction in the presence of varying amounts of Anabaena thioredoxin. A. Kₚ of 2.0 μM was calculated from the reaction of Anabaena thioredoxin with the homologous reductase in the presence of 0.7 μM dithiothreitol.

Anabaena thioredoxin-S is also a substrate for E. coli NADPH-thioredoxin reductase as demonstrated in Fig. 3. The Kₚ of 2.0 μM was calculated from the reaction of Anabaena thioredoxin-S with the homologous reductase shown in Fig. 3. A summary of the purification procedure for Anabaena thioredoxin is shown in Table 3.
Chymotrypsin digestion and identification of cysteine residues were used to identify a tryptic peptide map of Anabaena thioredoxin (Fig. 4). Tryptic peptide maps of the native protein were obtained with purified E. coli thioredoxin. The corresponding peptide maps of Anabaena thioredoxin were obtained with both tryptic and the half-cystine-specific enzymes.

Amino acid composition and spectrophotometry. The amino acid composition of Anabaena thioredoxin is shown in Table 2. The content of E. coli (25) and calf liver (25) thioredoxins are included for comparison. The amino acid composition of Anabaena thioredoxin was determined by A280 and is shown in Table 2.

Fluorescence spectra. The fluorescence spectra of oxidized and reduced Anabaena thioredoxin are shown in Fig. 5. The fluorescence of oxidized thioredoxin is shown in the top panel, while the fluorescence of reduced thioredoxin is shown in the bottom panel. The fluorescence spectra of oxidized and reduced E. coli thioredoxin are shown in the legend to Fig. 4. The spectra were recorded at pH 7.0, and the fluorescence was measured at 300 nm. The fluorescence of oxidized thioredoxin was approximately 3-fold lower than the fluorescence of reduced thioredoxin.

Immunological cross-reactivity. Thioredoxin from Anabaena exhibits cross-reactivity with antibodies to E. coli thioredoxin (Fig. 6). As determined by the inhibition of enzyme activity, Anabaena thioredoxin reacted with E. coli thioredoxin at a 100% level. However, the results show only 10% cross-reactivity suggesting that a small homology exists in the antigenic determinant site(s) of the two proteins.

### Table 2: Amino Acid Composition of Anabaena Thioredoxin

| Amino Acid | Anabaena | E. coli (25) | Yeast (25) | Calf Liver (25) |
|------------|----------|-------------|-----------|----------------|
| Lys        | 10       | 10          | 12        |                |
| His        | 1        | 1           | 0         |                |
| Arg        | 9        | 1           | 1-2       |                |
| Asp        | 15       | 15          | 10        |                |
| Thr        | 2        | 4           | 5         |                |
| Ser        | 1        | 1           | 2         |                |
| Gly        | 6        | 6           | 5         |                |
| Ala        | 12       | 12          | 14        |                |
| Val        | 3        | 4           | 3         |                |
| Pro        | 5        | 5           | 5         |                |
| Met        | 2        | 1           | 2         |                |
| Leu        | 13       | 15          | 13        |                |
| Ile        | 2        | 2           | 2         |                |
| Tyr        | 2        | 2           | 2         |                |
| Tryp       | 2        | 1           | 1         |                |
| Total      | 106      | 108         | 105-111   | 103           |

Tryptic peptide maps. Tryptic peptide maps of [14C]carboxymethylated Anabaena and E. coli thioredoxin (Fig. 4) showed large differences in pattern compatible with the marked differences in total composition. However, the electrophoresis showed that the active center tryptic peptide from both proteins was identical in mobility and formed a long streak on chromatography. This indicated a similar composition and size of the active center peptide in both proteins (25).

Fig. 4. a) Peptide maps of tryptic digest of [14C]carboxymethylated Anabaena and E. coli thioredoxin. The tryptic peptide was radioactive. b) Identification of CYCL-D-PHE-TYR-CHE-lype in the active center of both thioredoxin. The tryptic peptides of E. coli thioredoxin and Anabaena thioredoxin were isolated bySephadex G-25 chromatography and ethanol precipitation. Reactions and separations see Experimental Procedures. Autoradiography showed only one radioactive peptide (B) detected on each map.

**Active center sequence of Anabaena thioredoxin.** The sequence of the active center was determined directly by the identity of a peptide obtained by combined digestion with trypsin plus cyanogen bromide of [14C]carboxymethylated thioredoxin (Fig. 4). This gives the peptide CYCL-14C-PHE-14C-TYR-CHE-lype. The reaction was started by adding purified E. coli thioredoxin or yeast thioredoxin. The Anabaena protein contains only one cystine-S-S-bridge. The UV-visible spectrum of Anabaena thioredoxin at pH 6.0 showed an absorption maximum at 280 nm. No additional chromophoric groups were detected and the molar extinction coefficient at 280-310 nm was calculated to be 13,330 M⁻¹ cm⁻¹ from amino acid analysis. The identity of the disulfide and aromatic amino acid content in Anabaena and E. coli thioredoxin was shown by cyanogen bromide cleavage (Fig. 1). Synthetic peptide fragments of Anabaena thioredoxin equivalent to thioredoxin (15,27,31) were used to determine the sequence.

The spectrum of oxidized thioredoxin-S₂ with dithiothreitol shows two peaks at 300 and 310 nm, corresponding to tryptophan and tyrosine emission, respectively. The tryptophan fluorescence increases approximately 3-fold on reduction of thioredoxin, and the corresponding fluorescence increases approximately 1-fold on reduction of thioredoxin with dithiothreitol. The wavelengths of maximum absorption and emission are similar to those previously reported for E. coli thioredoxin (25).

Fig. 5. Fluorescence emission spectrum of Anabaena thioredoxin. Thioredoxin (50 nmol) was suspended in 100 mM sodium phosphate buffer, pH 7.0, in a total volume of 250 μl. Thioredoxin was reduced by adding 1 mM dithiothreitol to the cuvette. Anabaena thioredoxin was 100% labeled as determined by absorption at 280 nm. Thioredoxin was then determined with E. coli thioredoxin reductase using DTNB as electron acceptor as described in the legend to Fig. 1. E. coli thioredoxin gave a maximum emission at 310 nm, and the other at 310 nm. As indicated, the active center tryptic peptide from both proteins was identical in mobility and formed a long streak on chromatography. This indicated a similar composition and size of the active center peptide in both proteins (25).

Fig. 6. Reaction of thioredoxin with antibody to E. coli thioredoxin. After each step was performed as described in the Methods, E. coli thioredoxin (25 nmol) was subjected to SDS-PAGE on a 4-16% gradient gel and the relevant region of the gel was cut out and treated with pronase. The residual thioredoxin activity was determined with E. coli thioredoxin reductase. The results show only 10% cross-reactivity suggesting that a small homology exists in the antigenic determinant site(s) of the two proteins.