JOURNAL OF BACTERIOLOGY, Apr. 1992, p. 2592–2598
0021-9193/92/082592-07$02.00/0

Vol. 174, No. 8

Activities of Two Dissimilar Thioredoxins from the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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Received 31 October 1991/Accepted 6 February 1992

Thioredoxin is a small redox protein that functions as a reducing agent and modulator of enzyme activity. A gene for an unusual thioredoxin was previously isolated from the cyanobacterium *Anabaena* sp. strain PCC 7120 and cloned and expressed in *Escherichia coli*. However, the protein could not be detected in *Anabaena* cells (J. Alam, S. Curtis, F. K. Gleason, M. Gerami-Nejad, and J. A. Fuchs, J. Bacteriol. 171:162–171, 1989). Polyclonal antibodies to the atypical thioredoxin were prepared, and the protein was detected by Western immunoblotting. It occurs at very low levels in extracts of *Anabaena* sp. and other cyanobacteria. No antibody cross-reaction was observed in extracts of eukaryotic algae, plants, or eubacteria. The anti-*Anabaena* thioredoxin antibodies did react with another unusual thioredoxin-glutaredoxin produced by bacteriophage T4. Like the T4 protein and other glutaredoxins, the unusual cyanobacterial thioredoxin can be reduced by glutathione. The *Anabaena* protein can also activate enzymes of carbon metabolism and has some functional similarity to spinach chloroplast thioredoxin f. However, it shows only 23% amino acid sequence identity to the spinach chloroplast protein and appears to be distantly related to other thioredoxins. The data indicate that cyanobacteria, like plant chloroplasts, have two dissimilar thioredoxins. One is related to the more common protein found in other prokaryotes, and the other is an unusual thioredoxin that can be reduced by glutathione and may function in glucose catabolism.

Many physiological processes in photosynthetic organisms are regulated by light. The effect can be an indirect regulation of enzyme activity due to light-initiated changes in intracellular pH, ion concentration, or reduced cofactors (30). Thioredoxin is a small (M_r, ~12,000) disulfide redox protein. In photosynthetic organisms, levels of the dithiol form are enhanced by light-driven increases in reduced NADP and ferredoxin. Thioredoxin, in turn, can function as a reducing agent in processes such as sulfur metabolism and deoxynucleotide synthesis. Thioredoxin is also an efficient protein disulfide reductase and can modulate enzyme activity via redox control (see reference 12 for a review).

In chloroplasts of higher plants there are two thioredoxin fractions that can regulate a variety of enzyme activities. Thioredoxin f reduces critical disulfide bridges in the Calvin cycle enzymes fructose-1,6-bisphosphatase (22) and phosphoribulokinase (28); this leads to enzyme activation in the light. Thioredoxin m acts on the chloroplast NADP-dependent malate dehydrogenase (MDH) by a similar mechanism (29). These thioredoxins have also been shown to regulate the activities of a number of other plant enzymes (4). A thioredoxin with amino acid sequence similarity to and the functional properties of an m-type thioredoxin has been characterized in the cyanobacteria *Anabaena* sp. strain PCC 7119 (13) and *Anacystis nidulans* R2 (*Synechococcus* sp. strain PCC 7942) (25). Since MDH activity is of minor importance in cyanobacteria and is not under redox control, this cyanobacterial thioredoxin will be referred to simply as T-1. We had previously reported the occurrence of two thioredoxin genes in the cyanobacterium *Anabaena* sp. strain PCC 7120 (1). Only one of these was characterized further. The gene codes for an unusual thioredoxin that activates spinach fructose-1,6-bisphosphatase (FBPase) and had been assumed to be the counterpart of the chloroplast thioredoxin f (10). However, this thioredoxin (designated *Anabaena* T-2) has very little amino acid sequence identity with any known thioredoxins (9). Polyclonal antibodies specific for T-2 were raised in chickens, and T-2 can be detected by immunoblotting in both *Anabaena* strains and other unrelated cyanobacteria. This protein is not found in eukaryotic organisms. Both T-1 and T-2 were isolated and partially purified from *Anabaena* sp. strain 7120 cells. The structures and activities of the two cyanobacterial thioredoxins are compared with each other and with their counterparts from higher plant chloroplasts. *Anabaena* T-1 is similar to spinach chloroplast thioredoxin m and *Escherichia coli* thioredoxin in structure and activity. Thioredoxin T-2 is a unique protein that may be more closely related to another group of disulfide redox proteins, the glutaredoxins.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT), 5,5′-dithiobis(1-nitrobenzoic acid), NADPH, bovine insulin, bovine serum albumin (BSA), yeast glutathione reductase type III, reduced glutathione, acrylamide, anti-rabbit and anti-chicken immunoglobulin alkaline phosphatase conjugates, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were from the Sigma Chemical Co. Hydroxyethyl disulfide (HED) was from the Aldrich Chemical Co. DEAE-Sephacel, Sephacryl S-100, and the Mono Q column were purchased from Pharmacia. YM-5 ultrafiltration membranes (molecular weight cutoff, 5,000) and Centricon-3 filters (molecular weight cutoff, 3,000) were obtained from Amicon Corp. [5-^{3}H]CDP was purchased from Dupont, NEN Research Products. Ecoscint A scintillation cocktail was from National Diagnostics, Inc.

E. coli thioredoxin and *Anabaena* sp. strain PCC 7119 and PCC 7120 thioredoxins (T-1 and T-2, respectively) were purified from *E. coli* strains containing plasmid-encoded thioredoxins as previously described (1, 20). Thioredoxin-glutaredoxin produced by bacteriophage T4 and a mutant protein (V15G;Y16P) with the active site sequence altered to that of *E. coli* thioredoxin (26) were a generous gift of Matti

Nikkola, Swedish Agricultural University, Uppsala. *E. coli* thioredoxin reductase was purified from an overproducing strain containing the *trxB* gene on a pUC plasmid vector (9a). The enzyme was purified on 2',5'-ADP-Sepharose and hexylamine-agarose columns (27). NADP-dependent MDH and FBPase were partially purified from spinach chloroplasts (34). Spinach chloroplast thioredoxins m and f were homogeneous preparations supplied by Peter Schürmann, University of Neuchâtal. *E. coli* ribonucleoside diphosphate reductase was a gift of Britt-Marie Sjöberg, University of Stockholm. Polyclonal antibodies to *Anabaena* sp. strain 7119 thioredoxin were raised in a rabbit as described previously (21).

Methods. *Anabaena* sp. strains PCC 7119 and PCC 7120 were grown on modified Bg 11 medium (2) at 25°C at a constant illumination of 60 μmol m^{-2} s^{-1} photosynthetically active radiation. *Anabaena* sp. strain 7120 was grown under nitrogen-fixing conditions on the same medium without $NaNO_3$. Batch cultures for thioredoxin purification were grown on Bg 11 medium supplemented with 1 g of Na_2CO_3 per liter. These cultures were aerated with a mixture of 5% CO_2 in air and grown at 670 μmol m^{-2} s^{-1} photosynthetically active radiation. *Synechococcus* sp. strain R2 (*A. nidulans* PCC 7942) was grown under similar conditions. *Synechocystis* sp. strains PCC 6803 L and G were obtained from L. McIntosh, Michigan State University. Strain G is especially adapted to grow on 5 mM glucose (35). *Synechocystis* strains were grown autotrophically as described above. For photoheterotrophic growth, the medium for strain G was supplemented with 5 mM glucose. *Euglena gracilis* Z UTEX 753 was grown photoautotrophically on Cramer-Myers medium (5). *Chlamydomonas* sp. strain 2137 was obtained from R. Spreitzer, University of Nebraska, and was grown photoautotrophically on Sager-Granick medium (15). *Chlorella pyrenoidosa* UTEX 251 was grown under similar conditions. Fresh spinach leaves were obtained from a local wholesaler. Algal cells were harvested by centrifugation and stored at −10°C until used. Cells were suspended in Tris-HCl buffer (pH 7.5)–1 mM EDTA and broken by treatment with ultrasound for approximately 15 min. The ribs of the spinach leaves were removed, and the leaves were ground in a Waring blender in medium containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 0.35 M sorbitol. After chloroplasts were filtered through cheesecloth, they were separated from the cytosolic fraction by centrifugation at 2,500 × *g* for 5 min. Chloroplasts were lysed by brief sonication. The various lysates were then heated to 65°C and cooled immediately. Precipitated protein was removed by centrifugation at 15,000 × *g* for 20 min. The amount of protein in extracts was estimated from the A_{260} and A_{280} (31). These extracts were used for immunoblotting without further treatment.

Polyclonal antibodies to *Anabaena* sp. strain 7120 thioredoxin (T-2) were raised in chickens. The birds were injected with approximately 150 μg of *Anabaena* sp. strain 7120 thioredoxin in Freund complete adjuvant. The thioredoxin was isolated from *E. coli* BH2021 containing the T-2 gene on a pUC9 plasmid (1). Boosters containing approximately 50 μg of protein were given at 4-week intervals for a total of 12 weeks. Antibodies were purified from eggs collected after 4 weeks by the procedure of Jensenius et al. (18).

The basic procedure for detecting thioredoxins by immunoblotting was adapted from that of Harlow and Lane (14). Thioredoxins were separated on native 15% polyacrylamide slab gels and transferred to nitrocellulose. After transfer, the paper was incubated with polyclonal antibodies to one of the *Anabaena* thioredoxins. The reactive bands were visualized by alkaline phosphatase staining.

Isoelectric focusing was done by using the LKB Ampholine system and premade gels from Pharmacia. Gels in the range of pH 4.0 to 6.5 were run with *E. coli* thioredoxin (pI 4.7) and *E. coli* glutaredoxin (pI 4.8) as standards (17).

Enzyme assays. T-1 was assayed during purification by monitoring the thioredoxin-catalyzed reduction of 5,5'-dithiobis(1-nitrobenzoic acid) in the presence of *E. coli* thioredoxin reductase and NADPH (32). The thioredoxin-catalyzed reduction of insulin by DTT was done as previously described (1). 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 1.0 ml. The reaction was initiated by the addition of thioredoxin to the reaction cuvette, and the increase in turbidity was monitored at 650 nm over 30 min. This reaction was used in the purification of T-2.

The ability of thioredoxins to activate the chloroplast FBPase was determined by monitoring the release of P_i in the presence of thioredoxin-activated enzyme (16). The activation of chloroplast MDH by thioredoxins was determined by monitoring the oxidation of NADPH in the presence of oxaloacetate and preincubated enzyme (36).

Ribonucleotide reductase (CDP reductase) was assayed by using [^{3}H]CDP as a substrate and chemically reduced thioredoxin (11). Glutaredoxin activity was determined by measuring the enzyme-catalyzed reduction of HED by glutathione in a coupled assay with glutathione reductase (17).

Purification of thioredoxins from *Anabaena* sp. strain 7120. The purification of thioredoxins from *Anabaena* sp. strain 7120 was modified from that previously used to purify T-1 from *Anabaena* sp. strain 7119 (11). Approximately 90 g (wet weight) of *Anabaena* sp. strain 7120 cells were thawed and suspended in 300 ml of Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. The cells were broken by ultrasound treatment (with a Heat Systems Sonifier) for 20 min. The mixture was centrifuged at 10,000 × *g* for 20 min, and the pellet was discarded. The supernatant was heated to 65°C in a boiling-water bath and cooled immediately in an ice-water slurry. The heated extract was centrifuged for 10 min at 10,000 × *g*, and the pellet was discarded. The supernatant was loaded onto a column of DEAE-Sephacel (16 by 3 cm) that was previously equilibrated with 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The column was washed with the same buffer, and fractions were collected. The A_{280} of the column effluent was monitored. When the A_{280} dropped back to the baseline, the column was eluted with a gradient of NaCl (0 to 0.2 M) in the above buffer. Fractions were collected, and activity was determined by both the thioredoxin reductase and insulin precipitation assays. Both activities were found in the same fractions, which eluted near the beginning of the gradient. These fractions were pooled and concentrated by ultrafiltration with a YM-5 membrane at 70 lb/in^2 of N_2.

The DEAE pool was applied to a column of Sephacryl S-100 (95 by 1.6 cm) and eluted with 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. Active fractions eluted after the main protein peak. Both activities were associated with the same protein fractions. These were concentrated as above, dialyzed against 10 mM Tris-HCl (pH 8.0), and purified further by fast protein liquid chromatography on a Mono Q column. The column was preequilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. Approximately 1.5 mg of protein from the previous step was loaded onto the column. The column was washed with the

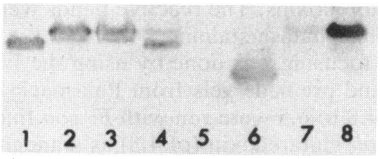

FIG. 1. Western immunoblot of cyanobacterial thioredoxins with polyclonal antibodies to *Anabaena* T-1. Polyclonal antibodies to T-1 were raised in a rabbit. These reacted strongly with T-1 from *Anabaena* sp. strain 7119 (lane 8, 0.2 μg of pure protein) and showed some cross-reaction with T-2 from *Anabaena* sp. strain 7120 (lane 7, 1 μg of pure protein). The T-1 antibodies also reacted with spinach chloroplast thioredoxin m (lane 6, 0.5 μg of pure protein) and *E. coli* thioredoxin (lane 5, 6 μg of pure protein). Crude extracts of *Anabaena* sp. strain 7119 (lane 4, 9 μg protein) and *Anabaena* sp. strain 7120 also showed two closely spaced bands (lane 3, 8 μg of protein from filaments without heterocysts; lane 2, 7 μg of protein from filaments with heterocysts). The thioredoxin from *Synechococcus* sp. strain R2 also cross-reacted with the antibodies (lane 1, 14 μg of protein in a crude cell extract).

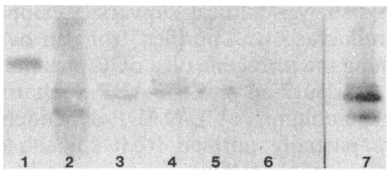

FIG. 2. Western immunoblot of thioredoxins with polyclonal antibodies to *Anabaena* T-2. Polyclonal antibodies to *Anabaena* T-2 were raised in a chicken. These reacted strongly with T-2 (lane 7, 0.5 μg of protein from *E. coli* containing the T-2 gene) but did not recognize T-1 (lane 6, 5 μg of pure protein). T-2 was detected in crude cell extracts of *Anabaena* sp. strain 7120 grown in the presence (lane 4, 14 μg of protein) or absence (lane 5, 18 μg of protein) of NO_3^-. T-2 was also found in crude cell extracts of *Anabaena* sp. strain 7119 (lane 3, 9 μg of protein) and *Synechococcus* sp. strain R2 (lane 2, 19 μg of protein). The antibodies also reacted strongly with thioredoxin-glutaredoxin from bacteriophage T4 (lane 1, 0.7 μg of protein).

above buffer and eluted with a shallow NaCl gradient (0 to 0.1 M). The A_{280} of the effluent was monitored.

RESULTS AND DISCUSSION

We had previously been unable to detect more than one thioredoxin fraction in *Anabaena* sp. strain 7120. Based on its cross-reaction with antibodies to *Anabaena* sp. strain 7119 thioredoxin (T-1) and reduction by *E. coli* thioredoxin reductase, we assumed that this fraction was the more common protein and that the unusual thioredoxin (T-2) was not expressed (1). However, as we showed in the earlier report, the proteins have similar mobilities in polyacrylamide gel electrophoresis (PAGE) with native gels at alkaline or neutral pH. The small difference in mobility expected for these thioredoxins is at the limit of resolution of routine PAGE. This was confirmed by isoelectric focusing experiments, in which T-1 and T-2 were found to have pIs of 6.0 and 5.3, respectively. Figure 1 shows a Western immunoblot of thioredoxins that react with polyclonal antibodies to T-1 from *Anabaena* sp. strain 7119. The antibodies recognize both *Anabaena* thioredoxins, although they react more strongly with T-1. As seen in lanes 7 and 8, the proteins have very similar mobilities under these conditions. These antibodies also cross-react with spinach thioredoxin m (lane 6) and very weakly with *E. coli* thioredoxin (lane 5). In crude extracts of *Anabaena* sp. strains 7119 and 7120, two bands (lanes 2, 3, and 4) that seem to correspond to T-1 and T-2 are partially resolved. The data suggest that both cyanobacterial strains produce both thioredoxins. The antibodies also react with a protein similar to T-1 in extracts of the unicellular cyanobacterium *Synechococcus* R2 (lane 1), which is known to have a homologous thioredoxin (25). Chicken polyclonal antibodies that recognize only the unusual T-2 were prepared. T-2 reacts strongly with these antibodies (Fig. 2, lane 7), whereas T-1 does not cross-react (lane 6). The multiple bands in lane 7 are caused by the deamination of one or more asparagine residues at the C terminus of T-2 (1). These antibodies also do not react with thioredoxins from *E. coli* or spinach chloroplasts (data not shown). With heat-treated cell extracts of *Anabaena* sp. strains 7119 and 7120, a faint band appears in the immunoblots at the position expected for T-2 (lanes 3, 4, and 5). Extracts of *Synechococcus* sp. strain R2 show two cross-reacting bands (lane 2), one at the position of *Anabaena* T-2 and a second band with slightly greater mobility corresponding to the deaminated form of *Anabaena* T-2. This antibody preparation also cross-reacted with thioredoxin (glutaredoxin) from bacteriophage T4 (lane 1). It can be concluded that the antibodies detect a T-2-type thioredoxin in both *Anabaena* strains and that thioredoxin levels are not significantly affected by growing the cyanobacteria under nitrogen-fixing conditions (*Anabaena* sp. strain 7120, lanes 3 and 4). The anti-T-2 antibodies cross-reacted with protein in extracts of the unicellular cyanobacterium *Synechocystis* sp. strain 6803 (Fig. 3, lanes 2, 3, and 4). These results (lanes 2 and 3) suggest that a T-2-type thioredoxin may be more abundant in cells grown without glucose. No anti-T-2-cross-reacting material was detected in extracts of *E. gracilis* Z (lane 1). Similarly, no reaction was observed with extracts prepared from *Chlamydomonas* sp. or *Chlorella* sp. (data not shown). It appears that the T-2-type thioredoxin is unique to cyanobacteria.

If, as indicated by the immunoblots, both thioredoxins are expressed in *Anabaena* sp. strain 7120, it should be possible to isolate both proteins from the cells. T-1 purification can be monitored as activity with *E. coli* thioredoxin reductase (20). T-2 is not reduced by the flavoprotein reductase, and activity is monitored as insulin precipitation (1). T-1 is also active in this reaction. Because of their similar size and charge, both proteins copurify through the first two steps of ion-exchange and size-exclusion chromatography. The two thioredoxins were separated by fast protein liquid chromatography on a Mono Q column with a shallow salt gradient (Fig. 4). The elution profile from the Mono Q column clearly shows two major peaks with some overlap. T-1 was approximately 85% pure after this step, whereas T-2 was still contaminated with

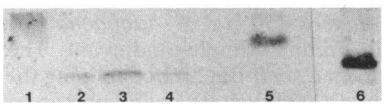

FIG. 3. Western immunoblot with polyclonal antibodies to *Anabaena* T-2. Polyclonal antibodies to T-2 were used to determine the presence of the unusual T-2 in other organisms. No cross-reacting material was found in a crude cell extract of *E. gracilis* Z (lane 1, 60 μg of protein). T-2 was present in extracts of *Synechocystis* sp. strain G grown in the presence (lane 2, 9 μg of protein) or absence (lane 3, 8 μg of protein) of 5 mM glucose and in *Synechocystis* sp. strain L (lane 4, 16 μg of protein). Lane 5 contained 0.6 μg of T4 thioredoxin-glutaredoxin.

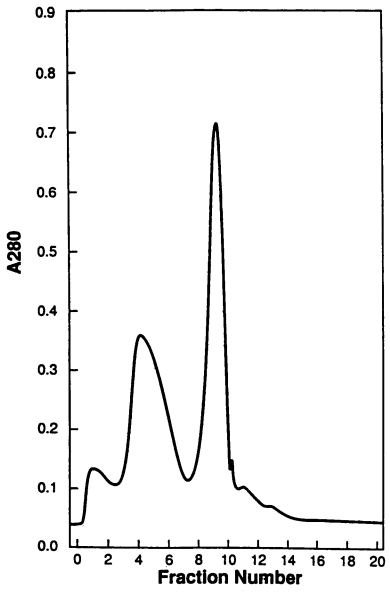

FIG. 4. Separation of *Anabaena* thioredoxins on ion-exchange chromatography. A partially purified thioredoxin fraction from *Anabaena* sp. strain 7120 (~1.5 mg of protein) was applied to a Mono Q HR5/5 column (Pharmacia). The column was eluted with a gradient of 0 to 100 mM NaCl in Tris-HCl (pH 8.0). Thioredoxin activity (insulin precipitation) was associated with both major peaks at fractions 5 and 9. Thioredoxin in the first peak could be reduced by NADPH and *E. coli* thioredoxin reductase, but that in the second peak did not react.

a small amount of T-1 and other proteins. Both fractions were active in the insulin precipitation assay. Activity with *E. coli* thioredoxin reductase was associated mainly with the first peak (T-1), whereas glutaredoxin activity was found only in peak 2 (T-2) (see below). The final yield of T-1 after this step was 5.3 mg from 90 g of cells; 1.6 mg of T-2 was obtained in the same preparation. A native gel showing the purification is shown in Fig. 5. T-1 was approximately 80% pure after chromatography on Mono Q (lane 5). T-2 was only approximately 60% pure after this step (lane 6). No additional purification of these fractions was attempted. As seen in Fig. 5, the electrophoretic mobilities of both cyanobacterial proteins on native gels were the same as those of the proteins isolated after gene cloning in *E. coli*.

In the previous report on thioredoxin from *Anabaena* sp. strain 7120, the gene was cloned into a pUC-type plasmid and the protein was expressed in *E. coli*. In the purification of this material, it was reported that the thioredoxin did not

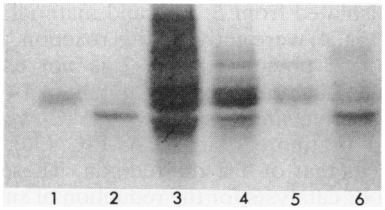

FIG. 5. Native PAGE of thioredoxins partially purified from *Anabaena* sp. strain 7120. Lanes: 1, 5 μg of cloned T-1; 2, 10 μg of cloned T-2; 3, DEAE pool, approximately 25 μg of *Anabaena* protein; 4, Sephacryl S-100 pool; 5, peak fraction 5 from Fig. 4, 8 μg of protein; 6, peak fraction 9 from Fig. 4, 15 μg of protein.

TABLE 1. Ability of thioredoxins to serve as substrates for *E. coli* thioredoxin reductase[a]

Thioredoxin	K_m (μM)	k_{cat} (min^{-1})	Catalytic efficiency (μM min^{-1})
E. coli thioredoxin	2.0	2,330	1,165
Anabaena T-1	17.0	2,330	137
Anabaena T-2	No reaction		
Spinach thioredoxin m	22.0	2,200	100
Spinach thioredoxin f	No reaction		

[a] The assay mixtures contained 100 mM Tris-HCl buffer (pH 8.0), 2 mM EDTA, 50 μg of BSA, 0.5 mM 5,5'-dithiobis(1-nitrobenzoic acid), and 0.24 mM NADPH in a final volume of 0.5 ml. Thioredoxins were added in a concentration range from 0.5 to 50 μM. The reaction was initiated by adding 13 nM *E. coli* thioredoxin reductase to the reaction cuvette. The increase in A_{412} was monitored at 25°C in a Hewlett-Packard 8450A double-beam spectrophotometer. The kinetic constants were determined from unweighted least-squares analyses of Lineweaver-Burk plots. *Anabaena* T-2 and spinach chloroplast thioredoxin f showed no activity up to 100 μM.

adhere to a DEAE-Sepharose column (1). Since the cyanobacterial thioredoxins do bind, a question arises as to whether the correct thioredoxin has been isolated. We have since found that the cloned thioredoxin is retained by both DEAE-Sepharose and Mono Q columns if the protein is reduced with DTT and 0.1 mM DTT is included in all buffers. We sequenced the N terminus of the cloned T-2 that adheres to the columns. The first 10 residues are identical to the T-2 sequence reported starting with the second methionine residue. These sequence results resolve any ambiguity concerning the start of transcription (1). Apparently, oxidized thioredoxin T-2 is not as stable as T-1 and readily forms intermolecular disulfide bonds at high concentrations, which causes the anomalous elution from ion-exchange columns. Purification is greatly facilitated by keeping the protein in the dithiol form.

We had previously reported that T-2 could not be reduced by NADPH and the flavoprotein thioredoxin reductase from *E. coli*, a property it has in common with spinach chloroplast thioredoxin f (Table 1). This is in contrast to T-1 and spinach Tm, which are good substrates for the heterologous reductase.

T-2 is a good activator for spinach chloroplast FBPase (Fig. 6). T-1 and *E. coli* thioredoxin are much less effective under similar reaction conditions. Approximately 1 μg of T-1 per μg of partially purified phosphatase preparation produced a doubling of activity. T-2, at approximately 0.2 μg/μg of phosphatase, produced a two- to threefold stimulation. T-2 however, will not activate spinach chloroplast NADP-dependent MDH under conditions in which T-1 and *E. coli* thioredoxin show stimulation (Fig. 7). The activity of *Anabaena* T-2 is comparable to that of spinach thioredoxin f in these assay systems, but it is not closely related to the plant protein. There is only 23% amino acid sequence identity between the cyanobacterial T-2 and chloroplast thioredoxin f. Other than the active site, only a few residues that are important in maintaining the tertiary structure are common to both proteins (9). The ability of either protein to reduce disulfide bonds in FBPase may be fortuitous, since other unrelated sulfhydryl-containing proteins (for example, purothionin [33]) can also serve this function.

The FBPase in cyanobacteria has not been completely characterized. In *Anabaena* sp. strain 7119, we found full activity in the presence of physiological concentrations of $MgCl_2$. Under these conditions, thioredoxin did not activate

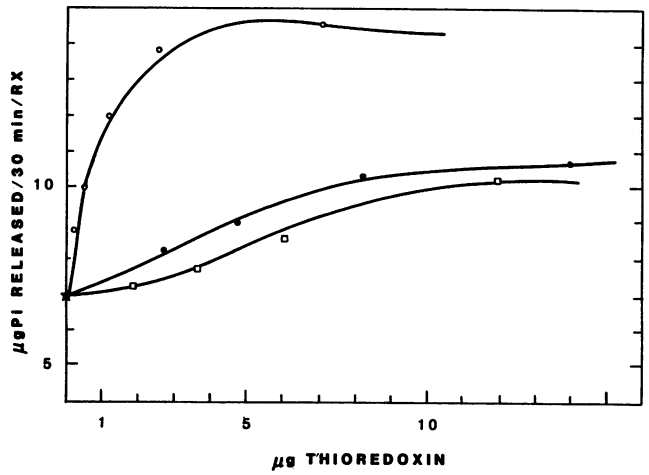

FIG. 6. Activation of spinach chloroplast FBPase by thioredoxins. The reaction mixtures contained 50 mM Tris-HCl buffer (pH 8.0), 12.5 mM DTT, 5 mM $MgCl_2$, 0 to 15 µg of thioredoxin, and 0.38 mg of partially purified spinach chloroplast FBPase in a final volume of 0.5 ml. The mixture was preincubated for 5 min at 25°C. The reaction was initiated by adding 3 mM fructose-1,6-bisphosphate. The reaction was stopped by adding 2 ml of Fiske-SubbaRow reagent, and the amount of P_i was determined from the A_{660} as compared with a standard curve. Symbols: ○, *Anabaena* T-2; □, *Anabaena* T-1; ●, *E. coli* thioredoxin.

the enzyme (34). Thioredoxins in plant chloroplasts can activate a number of enzymes in addition to those in the C-3 pathway. Other workers have shown that thioredoxin is very effective at reducing and deactivating glucose-6-phosphate dehydrogenase, which is the major enzyme of glucose catabolism in cyanobacteria (6). In contrast to the C-3 cycle enzymes, glucose-6-phosphate dehydrogenase would be turned off during the day when cells are photosynthesizing and reducing equivalents are high but would be activated in the dark. The results in Fig. 3 show that a heterotrophic *Synechocystis* strain grown on glucose has diminished levels of thioredoxin T-2, which is consistent with a role as negative effector of glucose catabolism.

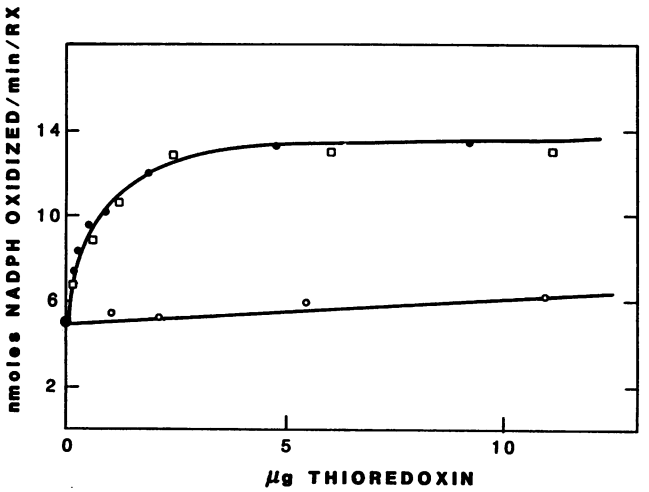

FIG. 7. Activation of spinach chloroplast NADP-MDH by thioredoxins. Reaction mixtures contained 80 mM Tris-HCl buffer (pH 8.0), 1.3 mM NADPH, 15 mM DTT, and 30 µg of partially purified spinach chloroplast NADP-MDH in a total volume of 0.5 ml. Thioredoxins were added from 0 to 15 µg per reaction. The mixtures were preincubated at 25°C for 10 min. The reaction was initiated by adding 5 mM oxaloacetic acid to the reaction cuvette. The oxidation of NADPH was monitored at 340 nm in a Hewlett-Packard 8450A spectrophotometer. ○, *Anabaena* T-2; □, *Anabaena* T-1; ●, *E. coli* thioredoxin.

TABLE 2. Thioredoxins as reducing agents for *E. coli* ribonucleotide reductase[a]

Thioredoxin	K_m (µM)	k_{cat} (min^{-1})	Catalytic efficiency (µM min^{-1})
E. coli thioredoxin	1.8	130	72
Anabaena T-1	2.0	130	65
Anabaena T-2	14.7	166	11.3
Spinach thioredoxin m	2.2	140	64
Spinach thioredoxin f	5.0	86	17
T4	8.3	81	9.7

[a] Reaction mixtures contained 80 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.6), 60 µg of BSA, 25 mM $MgCl_2$, 3 mM ATP, 1 mM DTT, 1.5 mM [^{3}H]CDP (approximately 20,000 cpm/nmol), and various concentrations of thioredoxins (0.5 to 15 µM) in a final volume of 0.05 ml. The reaction was initiated by adding 5.5 µg of *E. coli* ribonucleotide reductase and was incubated at 37°C for 10 min. The reaction was stopped by boiling. Deoxynucleotides were separated from substrate by chromatography on polyethyleneimine cellulose.

We previously reported that *Anabaena* T-2 is an effective reducing agent for ribonucleotide reductases from both *E. coli* and *Anabaena* sp. strain (7119) (1). This activity with *E. coli* ribonucleotide reductase is here compared with that obtained with thioredoxins from spinach and bacteriophage T4 (Table 2). All thioredoxins have the ability to transfer reducing equivalents to the reductase from DTT. However, the overall catalytic efficiencies for T-2 and thioredoxin f are comparable to that of T4 glutaredoxin, which is produced specifically as a reducing agent for the viral ribonucleotide reductase and has little activity with the host enzyme (3). From this comparison, it can be concluded that neither T-2 nor thioredoxin f functions as a reducing agent in this system in vivo.

Noting that polyclonal antibodies to T-2 also reacted with bacteriophage T4 thioredoxin, it seemed probable that the proteins have some functional as well as structural similarity. Bacteriophage T4 thioredoxin can be reduced by *E. coli* thioredoxin reductase and NADPH. It can also be reduced by glutathione and is almost as effective as *E. coli* glutaredoxin in catalyzing the reduction of small molecules such as hydroxyethyl disulfide. Since the viral thioredoxin has several structural features in common with glutaredoxins, it was proposed that the name of this protein be changed to T4 glutaredoxin (26). *Anabaena* T-2 can also be reduced by glutathione and will catalyze HED reduction (Fig. 8). Both cloned T-2 isolated from *E. coli* and material isolated from *Anabaena* (Fig. 4) were active in this reaction. From the data in Fig. 8, it is obvious that T-2 is not as effective as bacteriophage T4 glutaredoxin. A mutant of T4 glutaredoxin, in which the active site residues, C-V-Y-C, were changed to those found in thioredoxins, C-G-P-C (26), has activity comparable to that of T-2 thioredoxin. These proteins are relatively poor catalysts for the reduction of small molecules such as HED and do not bind reduced glutathione as well as glutaredoxin does. In separate experiments, it was determined that *Anabaena* T-2 cannot reduce ascorbate, although other thiol transferases (37) and the T4 protein can. Neither T-1, *E. coli* thioredoxin, nor the spinach chloroplast thiore-

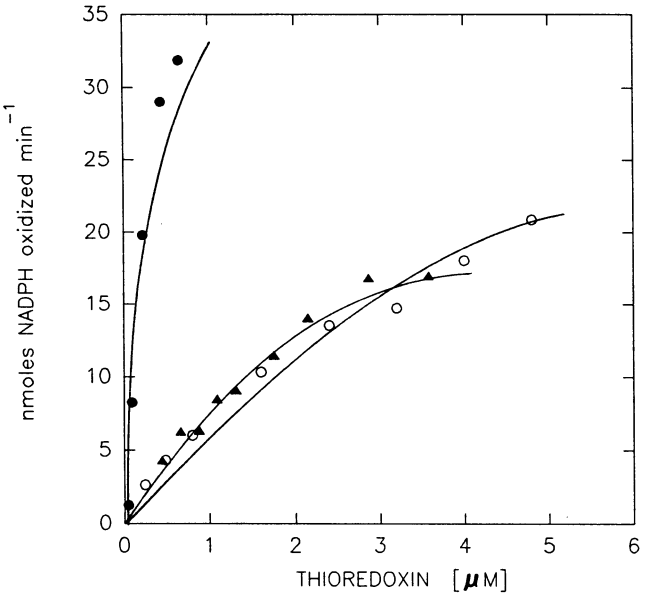

FIG. 8. Glutaredoxin activity of *Anabaena* T-2. Glutaredoxin, which is reduced by glutathione, catalyzes the reduction of hydroxyethyl disulfide. The reaction is monitored by the oxidation of NADPH by coupling to glutathione reductase. The assay mixtures contained 100 mM Tris-HCl buffer (pH 8.0), 2 mM EDTA, 50 μg of BSA, 0.3 U of yeast glutathione reductase, 0.75 mM HED, and 0.24 mM NADPH in a final volume of 0.5 ml. Reduced glutathione concentrations were varied between 0.1 and 5 mM. Reactions were initiated by adding 0 to 5 μM glutaredoxin or thioredoxin to the reaction cuvette. ●, T4 thioredoxin-glutaredoxin; ○, *Anabaena* T-2; ▲, T4 mutant thioredoxin V15G;Y16P.

doxins can be reduced by glutathione or catalyze HED reduction. Presumably, T-1 and T-2 are reduced in vivo by an NADPH-thioredoxin reductase or ferredoxin-thioredoxin reductase isolated from *Anabaena* sp. (8). Alternatively, glutathione may be the physiological reductant for T-2 when NADPH levels are high, i.e., in the light.

In addition to cyanobacteria, a number of other organisms have more than one thioredoxin. Two thioredoxins have been reported in *Corynebacterium nephridii* (23) and *Saccharomyces cerevisiae* (24). In these organisms, the two thioredoxins have a high degree of amino acid similarity and have similar functions. They may be the products of an evolutionarily recent gene duplication. Two thioredoxins are also found in chloroplasts of spinach (19) and *Chlamydomonas reinhardtii* (7). In these organisms, as in the cyanobacteria, the proteins are very different from each other and from any other known thioredoxins. They are most likely the products of a very early gene duplication, which presumably has occurred several times in the course of evolution. The T-1 type thioredoxin has been evolutionarily conserved and is homologous to the m-type spinach chloroplasts and to Ch2 in *C. reinhardtii*. It is also similar to the thioredoxin found in many other prokaryotic organisms (12). Unlike the thioredoxin in *E. coli*, this protein is essential in the autotrophic cyanobacteria (25). T-2, spinach chloroplast f, and *Chlamydomonas* Ch1 thioredoxins are highly divergent. It appears that the presence of two dissimilar thioredoxins is unique to the photosynthetic organisms, suggesting that the unusual thioredoxins play a specific role in photoautotrophic metabolism.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation grant DCB-9008136.

The assistance of Markku Saarinen and Alison Mall with antibody preparation is gratefully acknowledged.

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