A Large Chloroplast Thioredoxin *f* Found in Green Algae

Petra Langlotz, Wolfgang Wagner, and Hartmut Follmann

Fachbereich Chemie der Philipps-Universität, Biochemie, Hans-Meerwein-Straße, D-3550 Marburg, Bundesrepublik Deutschland

Z. Naturforsch. **41c,** 275–283 (1986); received October 25, 1985

Enzyme Activation, Fructose-bis-phosphatase, Ribonucleotide Reduction, *Scenedesmus obliquus*, Thioredoxin

Unicellular green algae differ from plant leaves in their thioredoxin profile. Besides several thioredoxins of regular size (M_r = 12,000), the heat-stable protein fraction of extracts from *Scenedesmus obliquus* cells contains a large protein of molecular weight M_r = 28,000 which is designated thioredoxin *f* on the basis of typical properties, in particular by its capacity to stimulate spinach chloroplast fructose-bis-phosphatase and, to lower degree, *E. coli* ribonucleotide reductase. The new thioredoxin was purified to apparent homogeneity by chromatography on DEAE cellulose, Sephadex G-50, CM cellulose, and Blue Sepharose. When tested in homologous enzyme systems, reduced thioredoxin *f* strongly activated algal fructose-bis-phosphatase, but was inactive towards the cytoplasmic algal ribonucleotide reductase; NADP malate dehydrogenase was also stimulated. The protein is missing in extracts from a chloroplast-free mutant strain, C-2A′, but appears together with other chloroplast components upon illumination. Protein *f* is therefore the main chloroplast thioredoxin of the green algae, probably corresponding to the smaller leaf chloroplast thioredoxins *f* and *m* combined. Algal thioredoxin *f* appears closely related, however, to the large thioredoxin found in a cyanobacterium, *Anabaena* sp.

Introduction

The presence of several thioredoxin polypeptides in plant cells is firmly established [1–4] but their intracellular distribution and functional differentiation are not fully understood. Most thioredoxins, regardless of their origin, have almost identical molecular weight (M_r = 11,000–12,000) and a highly conserved structure [5–8]. In contrast plant thioredoxins of the *f* type, which selectively activate chloroplast fructose-bis-phosphatase and are thought to participate in the light regulation of the Calvin cycle [9] appear to be a less homogeneous group. Thus, thioredoxin *f* activity of spinach chloroplasts has been separated into at least two components, f_A (M_r = 12,000) and f_B (M_r = 16,000–18,000) which is a dimeric protein [10, 11]. The cyanobacterium *Anabaena* sp. 7119 possesses an even larger thioredoxin *f* (M_r = 26,000) [12]; however the latter was reportedly unable to activate its endogeneous fructose-bis-phosphatase. It is of interest whether there exist further large members of the thioredoxin family and which activities they have in enzyme systems because a stringent thioredoxin definition is still missing.

Reprint requests to Prof. Dr. H. Follmann.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0300–0275 $ 01.30/0

We have analyzed the thioredoxin content of unicellular green algae *(Scenedesmus obliquus)* which, like plant seeds [4], contain three different polypeptides of the 12,000 dalton type [13, 14]. Algae offer the advantage that besides the thioredoxin-coupled activation of enzymes engaged in photosynthesis, nitrogen, and sulfur metabolism [15, 16] one can also study the classical thioredoxin function of hydrogen transfer in ribonucleotide reduction [13]. The latter reaction is barely measurable in higher plant tissues but the ribonucleotide reductase of *S. obliquus* has been characterized in detail [17]. We here show that in addition to thioredoxins of regular size the green algae also contain a large protein with typical properties of thioredoxin *f*.

Materials and Methods

Algal Cultures

Scenedesmus obliquus, strain D3, derived from stock cultures in the laboratory of Dr. H. Senger, Biology Department, Marburg, were grown for three days under continuous light in inorganic, aerated (3% CO_2) media at 28 °C [18]. Cells of mutant strain C-2A′ [19] were cultured in the dark in the same medium supplemented with 0.5% glucose and 0.25% yeast extract. Greening of mutant cells was produced by 24 hours illumination. Algae were harvested by

centrifugation and were resuspended twice in the buffer described below.

Materials and general methods

Proteins, enzyme substrates, and other chemicals were purchased from Merck, Serva, or Boehringer Mannheim, and radioactive cytidine diphosphate from Amersham-Buchler. DEAE cellulose (DE 32) and CM cellulose (CM 32) came from Whatman, Sephadex and Blue Sepharose gels from Pharmacia. Thioredoxin from *E. coli* was prepared by the published procedure [20]. Antibodies against *E. coli* thioredoxin coupled to Sepharose [21] were a gift from Dr. A. Holmgren, Karolinska Institutet, Stockholm. Protein was determined by the method of Lowry [22]. SDS polyacrylamide gel electrophoresis was carried out by standard procedures [23] on 15% gels in a pH 8.8 buffer system; proteins were stained with Coomassie Brilliant Blue R-250. Isoelectric focussing was performed on Ampholine PAG plates (LKB). Fluorescence spectra were recorded with a Shimadzu RF 502 spectrofluorimeter. For amino acid analysis, thioredoxin was reduced and carboxymethylated [24], hydrolyzed for 22 hours at 110 °C in 6 N HCl containing 0.1% phenol, and the sample analyzed on a Biotronic LC 7000 system.

Enzymes for thioredoxin assay

Enzymes were purified by published procedures, where available, and were assayed under optimum conditions for stimulation by added thioredoxins. Controls were done without thioredoxin and with the addition of *E. coli* thioredoxin.

Ribonucleoside diphosphate reductase of *E. coli* [25]: Substrates were 0.15 mM [5-^{3}H]CDP and 2 mM dithiothreitol. Incubation was for 30 min at 30 °C in 50 mM Tris buffer (pH 7.5) containing 15 mM Mg^{2+}, 0.8 mM EDTA, and 0.12 mM thymidine triphosphate as effector.

Ribonucleoside diphosphate reductase of *S. obliquus* [17]: Substrates, 4 μM [5-^{3}H]CDP and 1 mM dithiothreitol; incubation for 60 min at 30 °C in 50 mM K-phosphate buffer pH 6.7.

Ribonucleoside triphosphate reductase of *Anabaena* sp. [26]: Substrates, 0.1 mM [5-^{3}H]CTP and 1 mM dithiothreitol; coenzyme, 10 μM deoxyadenosylcobalamin. Incubation in the dark for 15 min at 37 °C in Hepes buffer (pH 8.2) containing 10 mM Ca^{2+}, 1 mM EDTA, and 0.05 mM deoxyadenosine triphosphate.

NADP malate dehydrogenase from spinach [2]: Preincubation of enzyme and thioredoxin proceeded for 30 min at 30 °C in 0.1 M Tris buffer (pH 7.9) containing 5 mM dithiothreitol and 0.25 mM NADPH. Enzyme assays were done in the same buffer with 2.5 mM oxaloacetate as substrate. NADP malate dehydrogenase from *S. obliquus* was prepared and assayed in analogy to the spinach enzyme.

Fructose-bisphosphatase from spinach [27] was purified up to the gel filtration step. Preincubation of enzyme and thioredoxin was for 5 min at 25 °C in 0.1 M Tris buffer (pH 7.9), containing 4 mM dithiothreitol and 1 mM Mg^{2+}. Enzyme assays were run by incubation with 6 mM fructose bisphosphate for 30 min at 30 °C.

Fructose-bisphosphatase from *S. obliquus* was prepared from algae which had been cultured for two days under continuous light and then for 12 hours in the dark. The extraction medium was 50 mM Tris buffer containing 5 mM EDTA, pH 7.85. The enzyme was precipitated at 40–90% ammonium sulfate saturation and then chromatographed over a column of Sephadex G-100 in 50 mM Tris buffer containing 1 mM EDTA, pH 7.5. Thioredoxin activation assays required incubation in the presence of 0.2 mM dithiothreitol and 4 mM Mg^{2+} in 0.1 M Tris buffer, pH 7.9, followed by determination of enzyme activity as described above.

Ferredoxin-dependent thioredoxin reductase from spinach chloroplasts: Enzyme assays were carried out in the laboratory of Dr. B. B. Buchanan, Dept. of Plant Molecular Biology, Berkeley, California, essentially as described [28].

Purification of thioredoxin f

All operations were performed at 0–5 °C. 50 g packed cells were suspended in 150 ml of 50 mM Tris-HCl buffer (pH 7.85) containing 5 mM mercaptoethanol and broken by 4 × 5 min agitation with glass beads (0.7 mm diameter) in a Bühler Vibrogen cell mill. Glass beads and cell debris were removed by filtration and centrifugation. To the extract was added streptomycin sulfate solution (4%) to give a final concentration of 0.8%, and after 15 min the precipitate was removed by centrifugation. The supernatant was adjusted to pH 4.3 by dropwise addition of cold 1 M acetic acid and the mixture cen-

trifuged for 5 min at 20,000 × *g*. After neutralization with 1 M ammonia the solution was kept for 5 min in a water bath at 60 °C, cooled rapidly, and centrifuged again.

The supernatant was filtered through a layer of glass wool and solid ammonium sulfate (0.68 g/ml) was added to 95% saturation. The pH was maintained at 7 by addition of concentrated ammonia. The precipitate, collected after 4 hours, was redissolved in a minimum volume of ammonium acetate buffer (0.02 M, pH 8.60, 5 mM mercaptoethanol), and freed from salt by gel filtration over a Sephadex G-25 column (5 × 30 cm) equilibrated with the same buffer. The protein fractions were applied to a column of DEAE cellulose (2.8 × 7.0 cm) equilibrated with the above ammonium acetate buffer. The column was eluted with a linear gradient of ammonium acetate pH 8.60 (0.02 M to 0.25 M, 5 mM mercaptoethanol) at a flow rate of 33 ml/h. Fractions with thioredoxin activity appeared shortly after the onset of the gradient and up to 0.17 M salt concentration. They were pooled and dialyzed against a neutral saturated ammonium sulfate solution. After 12 hours the precipitate was collected by centrifugation and dissolved in a small volume of Tris-HCl buffer (0.05 M, pH 7.85) containing 5 mM mercaptoethanol plus 10 mM dithiothreitol. After 1 hour the solution was layered on top of a Sephadex G-50 column (2.8 × 84 cm, equilibrated with the same buffer). At a flow rate of 16 ml/h two peaks of thioredoxin activity were eluted and pooled separately.

Thioredoxin *f*, of higher molecular weight, was dialyzed against sodium acetate buffer (0.01 M, pH 4.9, 5 mM mercaptoethanol) for 16 hours, reduced by the addition of 10 mM dithiothreitol and chromatographed on a column of CM cellulose (1.8 × 2 cm) equilibrated with the same buffer. Elution (24 ml/h) was carried out by addition of a linear sodium chloride gradient (0 to 0.1 M NaCl) to the acetate buffer. Lyophilization of the active material had to be avoided. When freezing thioredoxin *f* samples, it proved essential for the preservation of activity that solutions were rapidly frozen in liquid nitrogen and stored at −80 °C.

For chromatography of thioredoxin *f* on a Blue Sepharose column (1 × 8 cm) the protein sample was dialyzed against and applied in 10 mM Tris buffer containing 5 mM mercaptoethanol (pH 7.5). The column was then washed with several volumes of that buffer containing 0.2 M NaCl, and thioredoxin *f* was obtained by elution with 1.5 M NaCl in 10 mM Tris buffer. No specific binding of thioredoxin *f* occurred when algal thioredoxins were applied to a column (1.8 × 4 cm) of *E. coli* antithioredoxin-Sepharose [21] in 50 mM Tris-HCl buffer, pH 8.0 at 20 ml/h flow rate.

Results

Isolation and properties of a large algal thioredoxin

The small size of thioredoxins permits their preparative separation from most other proteins of a cell extract by gel permeation chromatography. Thioredoxin activity from *Scenedesmus obliquus*, capable of reacting with algal and bacterial ribonucleotide reductases [13], has been enriched by initial ammonium sulfate, heat, and ion exchange fractionation steps. When the active protein fraction which eluted from DEAE cellulose in a broad range was subjected to gel filtration on a column of Sephadex G-50 we observed, surprisingly, resolution into two well-defined activity peaks corresponding to 20,000−30,000 and 10,000−15,000 dalton molecular mass (Fig. 1a). The material contained in the latter protein peak constituted the normal (cytoplasmic) thioredoxin of M_r = 12,000 and could be separated into three isoproteins I−III, to be described elsewhere. The other thioredoxin activity of unusually large molecular weight was tentatively assigned a chloroplastic function because it could not be detected in a chlorophyll-free mutant strain (Fig. 1b). The column eluate has therefore been assayed with two independent enzyme systems, *viz.* ribonucleotide reductase from *E. coli* and spinach chloroplast fructose-bis-phosphatase. These readily available enzymes were preferred for practical reasons but the homologous, algal enzymes have also been used *(vide infra)*. It is seen in Fig. 1 that the larger protein fraction does indeed stimulate the Calvin cycle enzyme fructose-bis-phosphatase more efficiently whereas the thioredoxins of regular size are more active as hydrogen donors of ribonucleotide reduction, which is a cytoplasmic process. These two enzyme-activating effects are characteristic of any thioredoxin described so far; because of the selective stimulation of fructose-bis-phosphatase the new protein of M_r = 28,000 (see below) can be designated thioredoxin *f*.

Thioredoxin *f* was purified to apparent homogeneity by CM cellulose chromatography

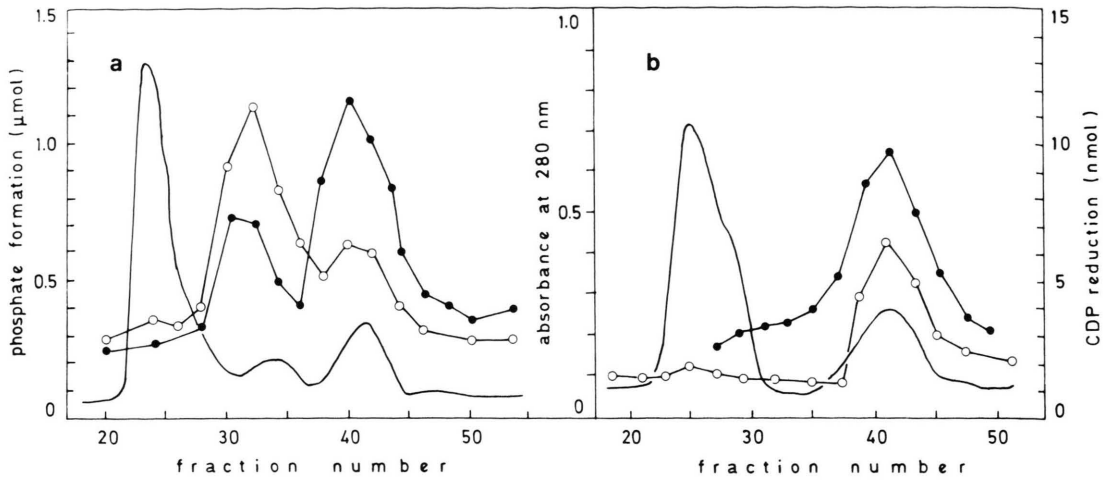

Fig. 1. Chromatography of thioredoxins from *Scenedesmus obliquus* on Sephadex G-50. a: Heat-stable protein extract from wild type cells; b: the same protein fraction isolated from the chloroplastfree mutant strain C-2A'. ●—●: Stimulation of ribonucleotide reductase (right scale); ○—○: activation of fructose-bis-phosphatase (left scale); —: protein (center scale).

(Fig. 2) and, if necessary, by a second gel filtration to remove contaminating smaller proteins. Thioredoxin *f* and the regular-size algal thioredoxins may also be separated by chromatography on Blue Sepharose, which selectively binds thioredoxin *f* and can then be eluted with 1.5 M NaCl. In all chromatography runs we have verified that the elution pattern remains unchanged after an extra reduction in 10 mM dithiothreitol, eliminating the possibility that the large protein is a dimeric thioredoxin linked by intermolecular disulfide bridges.

The purification of thioredoxins from *S. obliquus* extracts is summarized in Table I. These figures do not reflect the true concentration of either thioredoxin because the cell-free homogenate contains endogenous fructose-bis-phosphatase, and because thioredoxin *f* activity cannot be differentiated prior to separation of the individual species.

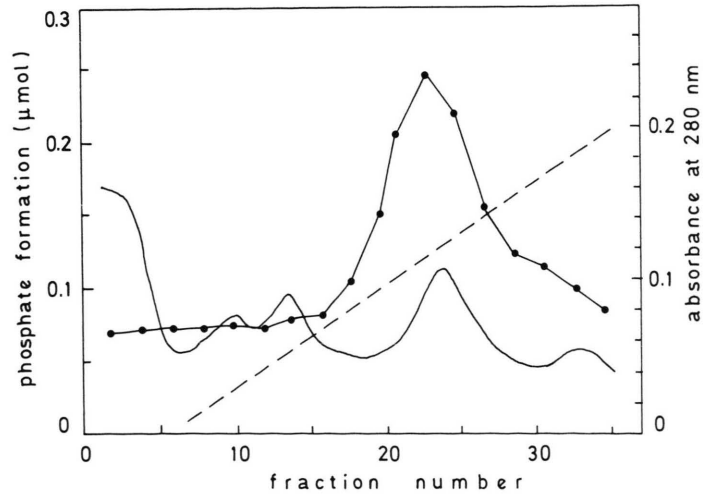

Fig. 2. Chromatography of thioredoxin *f* from *S. obliquus* on CM cellulose. —: Protein; --: NaCl concentration in the elution buffer (0–0.1 M). Thioredoxin was assayed by the stimulation of fructose-bis-phosphatase (left scale).

Table I. Purification of thioredoxins from an extract of *Scenedesmus obliquus*, prepared from 50 g packed cells. Activity cannot be measured in the initial homogenate because the test enzymes are inhibited by other components.

Purification step	Total protein [mg]	Total activity[a] reductase [μmol CDP]	FbPase [μmol PO_4]	Recovery reductase [%]	FbPase [%]
supernatant after acid precipitation	230	18.72	–	100	
supernatant after heat denaturation	94	13.44	–	72	
DEAE cellulose chromatography	36	9.16	485	49	100
Sephadex G50 chromatography					
thioredoxin *f*	3.6	0.5	226	4	47
other thioredoxins	3.0	5.34	200	29	41
CM cellulose chromatography					
thioredoxin *f*	0.35	–	54		11

[a] Calculation of specific activities is not meaningful in the purification of thioredoxins. Reductase: *E. coli* ribonucleotide reductase; FbPase: spinach fructose-bis-phosphatase used as test enzyme.

Properties

Electrophoresis in SDS-containing polyacrylamide gels indicates a molecular weight of M_r = 28,000 for thioredoxin *f* (Fig. 3). Our previous estimate of M_r = 23,500, using an impure preparation (protein "A") and obtained by gel filtration [14], was in error. The amino acid composition (with the exception of tryptophane) listed in Table II adds up to 241 residues and a calculated molecular weight of M_r = 29,800. A high number of aspartate and glutamate residues may explain the acidic isoelectric point of thioredoxin *f*, pI = 5.2, which was determined by isoelectric focussing.

The algal protein shares heat and acid stability with all other known thioredoxins; for example, heating to 90 °C for 5 minutes reduces its enzyme-activating capacity only 25%. However, *f* activity is less stable than most thioredoxins during prolonged storage in solution and during slow freezing or lyophilization. The fluorescence spectrum of thioredoxin *f* has a broad maximum at 350 nm

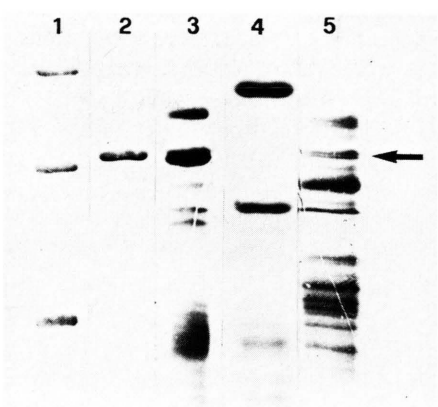

Fig. 3. Analysis of algal proteins on SDS-polyacrylamide (15%) gels. Lane 1: Marker proteins. From top: Ovalbumin, M_r = 45,000; chymotrypsinogen, M_r = 25,000; cytochrome *c*, M_r = 12,500. 2: Thioredoxin *f* purified by CM cellulose chromatography. 3: Thioredoxin *f*-containing protein fraction, partially purified by Sephadex G-50 chromatography, from wild type *S. obliquus*. 4: The same protein fraction isolated from mutant C-2A′ cells. 5: The same protein fraction isolated from a parallel culture of mutant C-2A′ after 24 hours of illumination. The arrow indicates the position of thioredoxin *f* (M_r = 28,000).

Table II. Amino acid composition of thioredoxin *f* from *S. obliquus*.

Amino acid	No. of residues	Amino acid	No. of residues
Ala	27	Lys	20
Arg	8	Met	4
Asx	25	Pro	12
Cys[a]	3	Phe	8
Glx	27	Ser	17
Gly	26	Thr	18
His	2	Trp	not determined
Ile	6	Tyr	7
Leu	19	Val	12

[a] Determined as S-carboxymethyl cysteine.

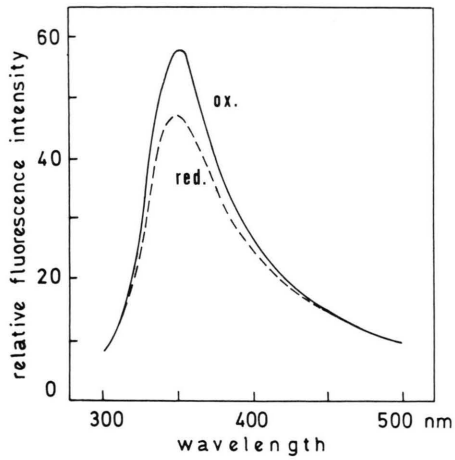

Fig. 4. Fluorescence spectrum of algal thioredoxin *f*. Excitation wavelength, 280 nm; protein concentration, 200 $\mu g \cdot ml^{-1}$ 50 mM phosphate buffer, pH 7. For reduction, the solution was made 3 mM in dithiothreitol and the sample incubated in the cuvette for 15 min.

(Fig. 4); its intensity decreases when the oxidized protein is reduced by dithiothreitol. This is in contrast to the spectral behaviour of *E. coli* thioredoxin where a fluorescence increase is observed upon reduction [29].

Because all regular thioredoxins have a high degree of sequence homology and conserved three-dimensional structure [7] they will bind to immobilized antibodies against *E. coli* thioredoxin. Whereas the three M_r = 12,000 thioredoxins of *S. obliquus* do react with the antibody, thioredoxin *f* showed no such crossreactivity.

Intracellular distribution

The presumed chloroplastic and cytoplasmic localization of the M_r = 28,000 and M_r = 12,000 thioredoxin species could not be directly demonstrated in wild-type algae because it is not possible to prepare intact chloroplasts from *S. obliquus* cells. The distribution was nevertheless clearly established by a comparison of the wild-type thioredoxin profile with that of a yellow mutant, C-2A′. These algae lack chloroplast structures when grown heterotrophically in the dark but develop a normal photosynthesis apparatus in the light [30]. As shown in Fig. 1b, gel filtration of a mutant cell protein extract did not resolve a heavier thioredoxin fraction besides the regular-size activity peak. Gel electrophoretic analysis of a "blind" preparation of thioredoxin *f* revealed, among other changes, the complete absence of a protein band at M_r = 28,000 (Fig. 3). The mutant did contain, however, all three M_r = 12,000 thioredoxins at normal levels (not shown). Wildtype algae were also grown in a heterotrophic, dark culture for control and had an unchanged thioredoxin profile including species *f*. Finally, mutant cells produced thioredoxin *f* as well as chlorophyll and other chloroplast components when illuminated for 24 hours (Fig. 3, lane 5).

Reactivity in enzyme systems

Distinctly different biochemical activities of thioredoxin *f* and of the regular thioredoxins of *S. obliquus* were noted during column chromatography, described above, and in a previous comparative study of multiple thioredoxin functions [14]. Great care must be taken to make functional, physiological assignments from such *in vitro* data because reduced thioredoxins interact with different enzymes by different mechanisms and because measured activities may depend critically upon proper assay conditions. Moreover, although thioredoxin and thioredoxin-linked enzymes often function well in heterologous combinations, unexpected species specificities have been observed among the plant proteins [31]. We have therefore prepared NADP-dependent malate dehydrogenase and alkaline Mg^{2+}-dependent fructose-bis-phosphatase from the green algae; both photosynthesis enzymes have not been described in this organism. The purified ribonucleoside diphosphate reductase of *S. obliquus* [17] was also tested for its reaction with algal thioredoxins.

The data summarized in Table III confirm the chloroplastic function of thioredoxin *f*. In homologous *in vitro* systems it did not react with the cytoplasmic enzyme of deoxyribonucleotide biosynthesis but activated NADP-MDH and strongly stimulated algal fructose-bis-phosphatase. Thioredoxin dependence of the latter enzyme (Fig. 5) required carefully controlled magnesium ion and thiol concentrations. These clearly differentiable activities become obscured in heterologous enzyme assays where, for example, thioredoxin *f* is able to support substrate reduction by deoxyadenosylcobalamin-dependent cyanobacterial ribonucleotide reductase [26] but is not very active with spinach chloroplast MDH. The algal cytoplasmic thioredoxins (similar to the bacte-

Table III. Activation of plant and bacterial enzymes by thioredoxins. Enzyme activities were determined under individually adjusted optimum conditions at saturating thioredoxin concentration. Absolute thioredoxin activities are calculated from thioredoxin-stimulated standard assays by subtraction of the control (-thioredoxin) values.

Enzyme	Relative activity [%] Without thioredoxin	+ Thioredoxin *f*	+ Thioredoxin M_r = 12,000	+ *E. coli* thioredoxin
from Scenedesmus obliquus:				
ribonucleotide reductase	20	inactive	100[a]	50
fructose-bis-phosphatase	10	100[b]	40	90
NADP malate dehydrogenase	15	100[c]	active[d]	active[d]
from other sources:				
E. coli ribonucleotide reductase	20	40	100[e]	150
Anabaena ribonucleotide reductase	40	80	100[f]	120
spinach fructose-bis-phosphatase	5	100[g]	55	80
spinach NADP malate dehydrogenase	10	20	100[h]	100

[a] Corresponds to 17 μmol CDP reduction·mg^{-1}; [b] 2.5 μmol phosphate liberation·mg^{-1}; [c] 2.5 μmol NADPH oxidized·min^{-1}·mg^{-1}; [d] complex kinetics (no saturation concentration); [e] 1.2 μmol CDP reduction·mg^{-1}; [f] 60 nmol CTP reduction·mg^{-1}; [g] 19 μmol phosphate liberation·mg^{-1}; [h] 10 μmol NADPH oxidized·min^{-1}·mg^{-1}.

rial protein included in Table III) exhibit generally lower specificity. Their outstanding activity in the algal ribonucleotide reductase system and very low activity with endogenous fructose-bis-phosphatase, however, once again substantiate the intracellular functional specialization of thioredoxins in *S. obliquus*.

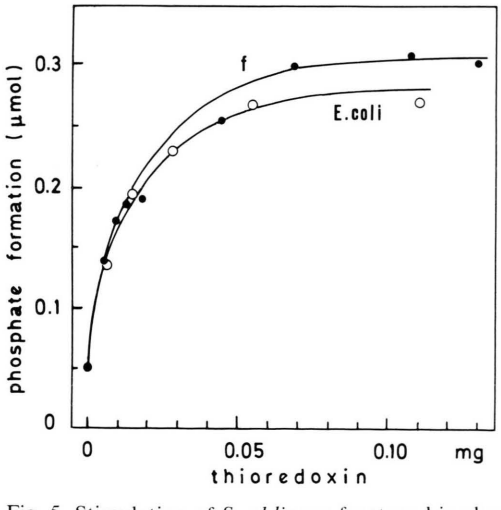

Fig. 5. Stimulation of *S. obliquus* fructose-bis-phosphatase by thioredoxins. ●: Thioredoxin *f* from *S. obliquus*; ○: thioredoxin from *E. coli*. The enzyme was assayed at pH 7.9 in the presence of 0.2 mM dithiothreitol and 4 mM magnesium ions.

We have not as yet been able to detect a thioredoxin reductase in the green algae. Presence of an NADPH-dependent enzyme may be ruled out because affinity chromatography on 2′,5′-ADP Sepharose readily yielded NADPH-glutathione reductase but not any thioredoxin-specific activity. Ferredoxin-thioredoxin reductase [28] was also not detectable under our assay conditions but its existence cannot be excluded. Oxidized algal thioredoxin *f* was instead tested with a ferredoxin-dependent enzyme preparation from spinach chloroplasts and was indeed reduced as efficiently as thioredoxin *f* from spinach leaves. In contrast, no reductioin occurred with NADPH and the thioredoxin reductase isolated from *E. coli* or from wheat seeds [4].

Discussion

The 28,000 dalton protein from *Scenedesmus obliquus* specified as thioredoxin *f* in this study is unique among all plant thioredoxins by its size. Other protein fractions of the 20,000 to 30,000 dalton category have been ascribed thioredoxin activity in plant extracts [32] but no such fraction has been purified and characterized in detail. We classify the large chloroplast protein a thioredoxin because it is completely analogous to 12,000 dalton thioredoxins in stability and reactivity. Structural studies are needed to show the presence of the conserved Cys-Gly-Pro-Cys active site or a related sequence in the new thioredoxin

f species. In view of the lack of immunological relationship with the most typical thioredoxin of *E. coli* it is clear that homologies, if any, may be distant.

On the other hand thioredoxin *f* of the green algae appears entirely homologous to the protein of M_r = 26,000 recently isolated from the filamentous cyanobacterium, *Anabaena sp.* 7119 [12]. The two proteins have closely comparable molecular weight and amino acid composition. They also share the same type of fluorescence spectrum, affinity to chromatography media of the Blue Sepharose type, inactivity with their endogenous ribonucleotide reductases (which will accept M_r = 12,000 thioredoxins in either case), high activity towards chloroplast fructose-bis-phosphatase, and moderate reactivity with NADP malate dehydrogenase. A difference lies in the apparent lack of stimulation of *Anabaena* fructose-bis-phosphatase by *Anabaena* thioredoxin *f* but this inactivity could well be due to inappropriate conditions, in particular too high a dithiothreitol concentration used in the cyanobacterial assay system.

In contrast to higher plants the unicellular algae have but one chloroplastic thioredoxin. Four or even more thioredoxins of the *f* type (M_r = 12,000; 18,000) and *m* type (malate dehydrogenase stimulating, M_r = 11,000) [3, 11, 33] have been found in leaves. Algal thioredoxin *f* resembles the *f* type species much more than the *m* proteins, *e.g.* in enzyme activation and affinity towards Blue Sepharose. As for physiological functions, it is conceivable that the large, single thioredoxin in algal chloroplasts serves to activate both these enzymes. Thioredoxin effects have also been noted in porphyrin biosynthesis [34, 35] and these effects should also be amenable to closer study in the greening mutant cells during chlorophyll synthesis. Although the regular cytoplasmic thioredoxins of *S. obliquus* (M_r = 12,000) activate NADP malate dehydrogenase *in vitro* (*cf.* Table III) we advise against "thioredoxin *m*" typification of these proteins because they most likely have their main function in deoxyribonucleotide biosynthesis.

Taken together these properties of cyanobacterial, algal, and higher plant thioredoxins may provide an explanation for the multitude of thioredoxins in plants, which is unparalleled in animals and other non-photosynthetic organisms. We hypothesize that a large cyanobacterial SH protein was the precursor of the large chloroplast thioredoxin present in green algae which in turn is the functional equivalent and possibly a precursor of the two smaller, specialized chloroplast thioredoxins found in higher plants. Additional thioredoxins of chloroplastic and/or of cytoplasmic localization in plant cells would be descendants of the regular, small polypeptides present in both the endosymbiont and the primeval host cell. Such relationships, which are in accord with the endosymbiont theory of cell organelles, can obviously be tested as plant thioredoxin amino acid sequences become available.

Acknowledgements

This work has been supported by grants from Deutsche Forschungsgemeinschaft, Fo 50/12, 13 and Sonderforschungsbereich 305. We thank Drs. B. Buchanan, University of California, Berkeley, Florence Gleason, University of Minnesota, Navarre, and H. Senger, Marburg, for providing enzyme samples and algal cultures. The assistance of G. Schimpff-Weiland and Dr. R. Hofmann in ribonucleotide reductase assays is also gratefully acknowledged.

[1] R. A. Wolosiuk, N. A. Crawford, B. C. Yee, and B. B. Buchanan, J. Biol. Chem. **254,** 1627–1632 (1979).
[2] J. P. Jacquot, J. Vidal, P. Gadal, and P. Schürmann, FEBS Lett. **96,** 243–246 (1978).
[3] P. Schürmann, K. Maeda, and A. Tsugita, Eur. J. Biochem. **116,** 37–45 (1981).
[4] A. Berstermann, K. Vogt, and H. Follmann, Eur. J. Biochem. **113,** 339–344 (1983).
[5] M. Meng and H. P. C. Hogenkamp, J. Biol. Chem. **256,** 9174–9182 (1981).
[6] A. Tsugita, K. Maeda, and P. Schürmann, Biochem. Biophys. Res. Commun. **115,** 1–7 (1983).
[7] H. Eklund, C. Cambillau, B.-M. Sjöberg, A. Holmgren, H. Jörnvall, J. O. Höög, and C.-I. Bränden, EMBO J. **3,** 1443–1449 (1984).
[8] F. K. Gleason, M. M. Whittaker, A. Holmgren, and H. Jörnvall, J. Biol. Chem. **260,** 9567–9573 (1985).
[9] B. B. Buchanan, Ann. Rev. Plant Physiol. **31,** 341–374 (1980).
[10] J. M. Soulié, J. Buc, J. C. Meunier, J. Pradel, and J. Ricard, Eur. J. Biochem. **119,** 497–502 (1981).
[11] J. Buc, M. Rivière, B. Gontero, P. Sauve, J. C. Meunier, and J. Ricard, Eur. J. Biochem. **140,** 199–202 (1984).
[12] M. M. Whittaker and F. K. Gleason, J. Biol. Chem. **259,** 14088–14093 (1984).
[13] W. Wagner and H. Follmann, Biochem. Biophys. Res. Commun. **77,** 1044–1051 (1977).
[14] W. Wagner, H. Follmann, and A. Schmidt, Z. Naturforsch. **33c,** 517–520 (1978).
[15] R. Tischner and A. Schmidt, Plant Physiol. **70,** 113–116 (1982).
[16] J. D. Schwenn and U. Schriek, FEBS Lett. **170,** 76–80 (1984).
[17] R. Hofmann, W. Feller, M. Pries, and H. Follmann, Biochim. Biophys. Acta **832,** 98–112 (1985).
[18] N. I. Bishop and H. Senger, Methods Enzymol. **23,** 53–66 (1971).
[19] H. Senger and N. I. Bishop, Plant Cell Physiol. **13,** 633–649 (1972).
[20] T. C. Laurent, E. C. Moore, and P. Reichard, J. Biol. Chem. **239,** 3436–3444 (1964).
[21] B. M. Sjöberg and A. Holmgren, Biochim. Biophys. Acta **315,** 176–180 (1973).
[22] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and F. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[23] U. K. Laemmli, Nature **227,** 680–685 (1970).
[24] A. M. Crestfield, S. Moore, and W. H. Stein, J. Biol. Chem. **238,** 622–627 (1963).
[25] N. C. Brown, Z. N. Canellakis, B. Lundin, P. Reichard, and L. Thelander, Eur. J. Biochem. **9,** 561–573 (1969).
[26] F. K. Gleason and T. D. Frick, J. Biol. Chem. **255,** 7728–7733 (1980).
[27] B. B. Buchanan, P. Schürmann, and P. P. Kalberer, J. Biol. Chem. **246,** 5952–5959 (1971).
[28] A. de la Torre, C. Lara, R. A. Wolosiuk, and B. B. Buchanan, FEBS Lett. **107,** 141–145 (1979).
[29] A. Holmgren, J. Biol. Chem. **247,** 1992–1998 (1972).
[30] N. I. Bishop and H. Senger, Plant Cell Physiol. **13,** 937–953 (1972).
[31] I. Häberlein, G. Schimpff-Weiland, and H. Follmann, Biochem. Biophys. Res. Commun. **127,** 401–406 (1985).
[32] N. A. Crawford, B. C. Yee, and B. B. Buchanan, Plant Sci. Lett. **22,** 317–326 (1981).
[33] N. A. Crawford, B. C. Yee, A. N. Nishizawa, and B. B. Buchanan, FEBS Lett. **104,** 141–145 (1979).
[34] J. D. Clement-Metral, FEBS Lett. **101,** 116–119 (1979).
[35] A. P. Balangé and C. Lambert, Plant Sci. Lett. **32,** 253–259 (1983).