Green Algae (*Scenedesmus obliquus*) Contain Three Thioredoxins of Regular Size

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A comprehensive thioredoxin profile of *Scenedesmus obliquus* has been established by chromatography of heat-stable protein extracts on five different ion exchange, gel permeation, and affinity chromatography columns and using three different assay systems including homologous *S. obliquus* ribonucleotide reductase, chloroplast fructose-bis-phosphatase, and NADP malate dehydrogenase. Four different thioredoxins were purified to homogeneity. Besides the large chloroplast thioredoxin *f* described previously, the algae contain three proteins of molecular weight 12,000 designated thioredoxin I, II, and III. They bind specifically to antibodies against *E. coli* thioredoxin. Chloroplast-free mutant algae (strain C-2A′) lack thioredoxin *f* but contain all three regular thioredoxins. Species I and II have very similar amino acid composition and enzyme-stimulating activities. They are considered cytoplasmic thioredoxins which serve as hydrogen donors in algal deoxyribonucleotide biosynthesis. Thioredoxin III is of low activity towards all the presently tested enzymes and its physiological role remains unknown; its role as a glutaredoxin could be excluded. All non-photosynthetic plant cells analyzed so far (mutant algae, seeds, and roots) contain a set of three regular-size thioredoxins.

Introduction

Studies on the multitude of thioredoxins in plants, discovered in the laboratories of Buchanan, Gadal, and ourselves [1–3], have focussed on chloroplastic localization and functions of the dicysteine polypeptides. For example, reduced *f* type and *m* type thioredoxins in the light selectively activate chloroplast fructose-bis-phosphatase and NADP malate dehydrogenase, respectively, and thereby link photosynthetic electron flow to light regulation of the Calvin cycle and related processes [4]. However, such chloroplast thioredoxins exhibit a wide range of molecular weights from M_r = 9,000 to 28,000 [1–3, 5–7] and form a much more heterogeneous group than the highly conserved thioredoxins of M_r = 12,000 isolated from all other organisms; accordingly their patterns are still not fully known. To estimate the total number and to determine the biochemical role of *all* thioredoxins in a plant extract it is necessary to also recognize the thioredoxin(s) of cytoplasmic origin and their light-independent functions in plants. Hydrogen transfer in reductive deoxyribonucleotide formation is, *inter alia*, such a thioredoxin-requiring process essential for cell proliferation in plants like in any other organism [8]. Presumed cytoplasmic thioredoxin fractions from leaves have been described [9] but were not characterized. We have purified to homogeneity three M_r = 12,000 thioredoxins from wheat and soy bean seeds [10] but their possible localization in plastid precursors has not been analyzed. It will usually be difficult in higher plants to identify cytoplasmic thioredoxins that correspond to the well-known proteins of bacteria or animal cells because no appropriate plant enzyme for direct determination has been recognized, and assays with heterologous enzymes may be misleading.

The presence of a large chloroplast thioredoxin *f* and smaller polypeptides of thioredoxin activity in *Scenedesmus obliquus* cells has recently been reported [3, 7]. Green algae have several advantages for the study of plant thioredoxins: Besides thioredoxin activation of chloroplast enzymes one can also measure the cytoplasmic, cell cycle-related ribonucleotide reaction, which is barely detectable in higher plants [11]. Heterotrophically growing

Abbreviations: CDP, cytidine 5′-diphosphate; MDH, malate dehydrogenase; SDS, sodium dodecyl sulfate.

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mutants of *S. obliquus* are available which lack chloroplast or proplastid structures and can thus be analyzed for other but chloroplast thioredoxins. Finally the eukaryotic unicellular algae should form a link between the simple content of two thioredoxins in cyanobacteria like *Synechococcus* and *Anabaena* species [12, 13] and the very complex thioredoxin pattern found in leaves. We have, therefore, purified to homogeneity and characterized the M_r = 12,000 thioredoxins present in *Scenedesmus obliquus* wild type cells and a non-green mutant strain. It appears likely that three such proteins constitute a basic set of thioredoxins in non-photosynthetic plant cells in general.

Materials and Methods

Algal cultures

Scenedesmus obliquus, strain D3, cells were grown in inorganic aerated (3% CO_2) media at 28 °C [14]. As the thioredoxin content in the algae is not cell cycle-dependent, cultures were continued for three days under constant illumination. Mutant C-2A′ cells were cultured as previously described [7].

Materials and general methods

Reagents and biochemicals were purchased from Merck, Serva, or Boehringer Mannheim, and radioactive cytidine diphosphate from Amersham-Buchler. Thioredoxin from *E. coli* was prepared by the published procedure [15]; glutaredoxin from *E. coli* and antibodies against *E. coli* thioredoxin coupled to Sepharose were generous gifts of Dr. A. Holmgren, Stockholm. DEAE cellulose and CM cellulose (DE 32, CM 32) came from Whatman, and Sephadex gels from Pharmacia. Protein was determined by the method of Lowry [16]. SDS polyacrylamide gel electrophoresis was carried out on 15% gels in a pH 8.8 buffer system, and native electrophoresis on 7.5% polyacrylamide gels at pH 9.5; isoelectric focussing was performed on Ampholine plates (LKB). Proteins were stained with Coomassie Brilliant Blue R-250, or by the silver procedure. Fluorescence spectra were recorded with a Shimadzu RF 502 spectrofluorimeter. Amino acid analyses of carboxymethylated [17] thioredoxins were done on a Biotronic LC 7000 system; protein hydrolysis took place in 6 N HCl, containing 0.1% phenol, for 22 h at 110 °C.

Purification of thioredoxins

Algae were harvested, homogenized in a cell mill, and the viscous, green extract subjected to streptomycin sulfate, acid (pH 4.3), heat (60 °C), and ammonium sulfate precipitation as described [7, 18]. After desalting on Sephadex G-25, proteins were chromatographed on a column (2.8 × 7 cm) of DEAE cellulose equilibrated in 0.02 M ammonium acetate buffer, pH 8.60, containing 5 mM mercaptoethanol. The column was first washed for 1 h with the dilute buffer and was then eluted with a linear gradient of 0.02 to 0.25 M ammonium acetate, pH 8.60, 5 mM mercaptoethanol, at a flow rate of 33 ml/h. One small activity peak was collected separately at about 0.08 M ammonium acetate concentration, and the main activity peak around 0.14 M salt concentration. The first material (thioredoxin III) was rechromatographed on DEAE cellulose under the same conditions. The two fractions were dialyzed for 12 h against neutral saturated $(NH_4)_2SO_4$ solution. The precipitates were collected by centrifugation and then redissolved in a small volume of 0.05 M Tris-HCl buffer, pH 7.85, containing 5 mM mercaptoethanol plus 10 mM dithiothreitol.

The main thioredoxin fraction (containing species *f,* I, and II) was chromatographed on a column (2.8 × 84 cm) of Sephadex G-50, equilibrated and operated in the above Tris-HCl buffer. At a flow rate of 16 ml/h, two peaks of thioredoxin activity were eluted corresponding to $M_r > 20{,}000$ and $M_r \approx 12{,}000$. The protein of larger molecular weight, thioredoxin *f,* was collected and purified further as described [7].

The thioredoxin species of lower molecular weight was dialyzed against sodium acetate buffer (0.02 M, pH 4.5, 5 mM mercaptoethanol) for 16 h, reduced by the addition of 10 mM dithiothreitol and adsorbed to a column of CM cellulose (1 × 8 cm). The column was eluted with a linear gradient of sodium acetate, pH 4.5 (0.01 M to 0.1 M, 5 mM mercaptoethanol) at a rate of 12 ml/h. Two peaks of thioredoxin activity, I and II, were pooled separately.

Minor thioredoxin III, after rechromatography on DEAE cellulose, was chromatographed on a column (2 × 53 cm) of Sephadex G-75 in the abovementioned Tris-HCl buffer. The column was run at a flow rate of 12 ml/h and thioredoxin activity was eluted in fractions corresponding to M_r = 10,000–20,000. For purification on a column (1.8 × 4 cm) of *E. coli* anti-

thioredoxin-Sepharose [19] the protein sample was applied in 50 mM Tris-HCl buffer, pH 8.0, and the column washed with 4 bed volumes of the same buffer. Elution of unspecifically bound proteins was carried out with 1 M Tris buffer, pH 8.0, the column washed again with dilute buffer, and thioredoxin was then eluted with 4 bed volumes of 0.1 M acetic acid (pH 2.2). The effluent was neutralized immediately with a solution of $NaHCO_3$. Thioredoxins I and II could be bound and eluted in the same way.

Enzymes for thioredoxin activity determination

Ribonucleoside diphosphate reductase from *E. coli,* prepared as in [20], was assayed in 50 mM Tris buffer, pH 7.5, containing 0.15 mM [5-^{3}H]CDP, 2 mM dithiothreitol, 0.12 mM thymidine triphosphate, 15 mM Mg^{2+}, and 0.8 mM EDTA. Incubation was for 30 min at 30 °C. Ribonucleoside diphosphate reductase of *S. obliquus* [11] was assayed in 50 mM K-phosphate buffer, pH 6.7, containing 4 μM [5-^{3}H]-CDP and 1 mM dithiothreitol, for 60 min at 30 °C. Deoxyadenosylcobalamin-dependent ribonucleoside triphosphate reductase of *Anabaena* sp. [21] was made available in the laboratory of Dr. F. K. Gleason, University of Minnesota, Navarre. Substrate reduction in the absence of added thioredoxins was subtracted from the activity in thioredoxin-containing assay mixtures.

Fructose-bis-phosphatase was purified from spinach leaves [22]. Enzyme and thioredoxin were preincubated for 5 min at 25 °C in 0.1 M Tris buffer, pH 7.9, containing 4 mM dithiothreitol and 1 mM Mg^{2+}, and enzyme activity was then determined with 6 mM fructose-bis-phosphate for 30 min at 30 °C. The enzyme of *S. obliquus* was prepared as described previously [7]; thioredoxin activation required 0.2 mM dithiothreitol and 4 mM Mg^{2+} in the above Tris buffer.

NADP malate dehydrogenase was obtained from spinach leaves as described [2]; the same procedure could be applied to enzyme purification from *S. obliquus* cells. Activation of enzyme by thioredoxins was done by 30–90 min preincubation at 30 °C in 0.1 M Tris buffer containing 5 mM dithiothreitol and 0.25 mM NADPH. Enzyme activity was then measured spectrophotometrically with 2.5 mM oxaloacetate as substrate.

Ferredoxin-dependent thioredoxin reductase from spinach chloroplasts was made available in the laboratory of Dr. B. B. Buchanan, University of California, Berkeley, and was assayed as described [23].

Results

Purification of three algal thioredoxins

The determination of a complete thioredoxin profile in a cell extract requires parallel use of several indicator enzymes of more general and of more narrow specificity. For routine measurements we have selected ribonucleotide reductase from *Escherichia coli,* NADP malate dehydrogenase from spinach, and fructose-bis-phosphatase from spinach for assaying thioredoxin activity in protein fractions from *Scenedesmus obliquus*. The first two are unspecific with regard to the origin of a stimulatory thioredoxin and closely resemble the algal enzymes in their properties, whereas fructose-bis-phosphatase is more specific for *f* type thioredoxins. Moreover the activity of *Scenedesmus* thioredoxins with the same three enzymes isolated from *Scenedesmus* has been determined because major quantitative and even qualitative differences in heterologous enzyme-thioredoxin mixtures are not uncommon. In combination these assay systems should detect virtually any stimulatory protein of thioredoxin nature in a photosynthetic organism even if individual fractions have low affinity for one or the other test enzyme.

Thioredoxin activity in cell-free *Scenedesmus* extracts becomes measurable only after initial reduction of the amount of macromolecular materials in the viscous homogenate by streptomycin, acid, and heat treatment [18]. Fractionation on DEAE cellulose yielded a broad, heterogeneous peak of enzyme-stimulating material eluting at around 0.14 M acetate concentration (Fig. 1). Coloured proteins eluted at higher salt concentration and stimulated fructose-bis-phosphatase but not ribonucleotide reductase (fractions 42–50 in Fig. 1). They were identified as algal cytochrome *c*-553 and oxidized ferredoxin by their absorption spectra; we found no evidence for thioredoxin activity when these fractions were purified further. Furthermore no other, potentially unusual or unstable thioredoxins were detected when the initial heat and acid treatments were omitted from the protocol.

The total thioredoxin eluate from DEAE cellulose contained four separate proteins. One minor species is represented by the first, small activity peak of the column eluate (fractions 21–26 in Fig. 1) which was

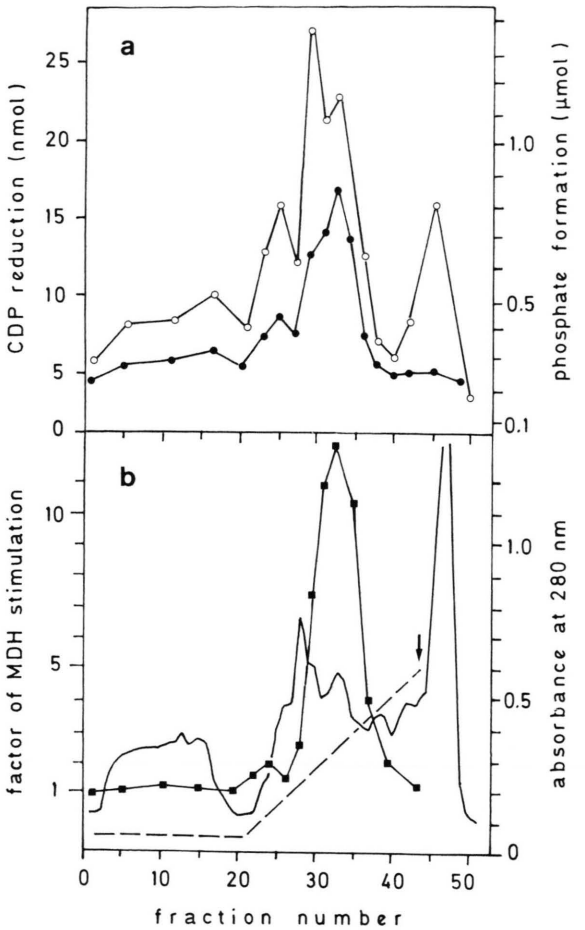

Fig. 1. Chromatography of protein extract from *Scenedesmus obliquus* on DEAE cellulose. The column was eluted with a gradient of 0.02–0.25 M ammonium acetate, pH 8.60 (dashed line); 1 M ammonium acetate was then applied to the column (arrow). Thioredoxin activity was assayed with *E. coli* ribonucleotide reductase (●——●; left scale in a); spinach fructose-bis-phosphatase (○——○); right scale in a); and spinach NADP malate dehydrogenase (■——■; left scale in b). Drawn line: protein absorption at 280 nm (right scale in b).

purified separately and was characterized as indicated below (thioredoxin III). The large, poorly resolved activity peak (fractions 27–40) was chromatographed on Sephadex G-50 and was separated into thioredoxin *f* (M_r = 28,000) described recently and a predominant activity peak of the expected molecular weight, M_r = 12,000 [7]. This material was further purified on CM cellulose where it separated again into two isoproteins, designated thioredoxin I and II (Fig. 2). Protein samples of

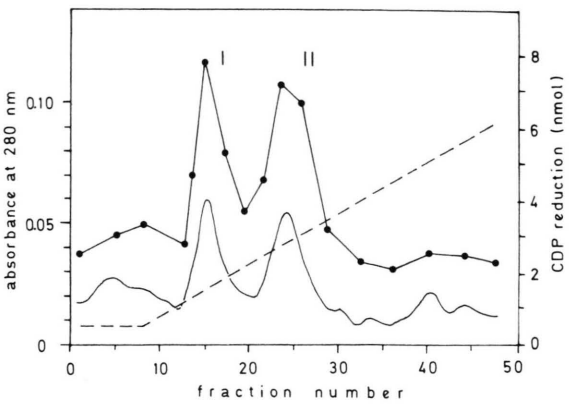

Fig. 2. Chromatography of algal thioredoxin I and II on CM cellulose. Thioredoxin fractions 37–47 from Sephadex G-50 were pooled and chromatographed. Elution was carried out with a 0.01–0.1 M sodium acetate buffer, pH 4.5 (dashed line). Thioredoxin was assayed with *E. coli* ribonucleotide reductase (●——●, right scale). Determination of MDH stimulation (not drawn) did not reveal further activity peaks.

apparent homogeneity on denaturing and native electrophoresis gels could be obtained in this way (Fig. 4).

Thioredoxin III, clearly separated from the other proteins by repeated DEAE cellulose chromatography and gel filtration, was more difficult to handle

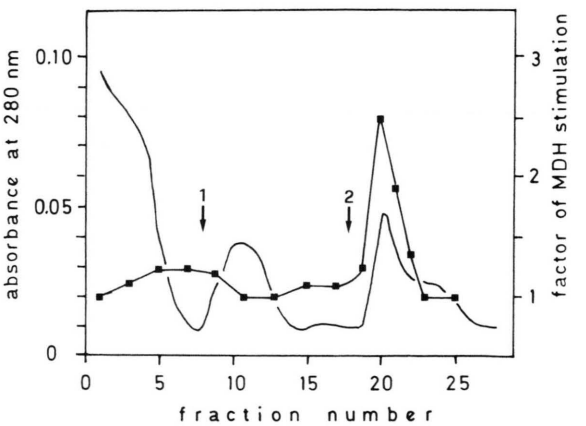

Fig. 3. Purification of algal thioredoxin III on antithioredoxin-Sepharose. Unspecifically bound proteins were eluted with 1 M Tris buffer, pH 8 (arrow 1), and thioredoxin with acetic acid/formic acid, pH 2 (arrow 2). Drawn line: protein absorption at 280 nm. ■——■: Thioredoxin activity measured by stimulation of NADP malate dehydrogenase (right scale).

because of its low enzyme activation and poor detectability in dilute column eluates. This species was best purified by binding to an *E. coli*-antithioredoxin Sepharose column and specific elution with acetic acid (Fig. 3). *Scenedesmus* thioredoxins I and II also bound tightly to the immobilized antibody whereas algal thioredoxin *f* had no affinity towards this material.

No further resolution of any of the thioredoxin fractions was observed in additional chromatography steps, *e.g.* on Blue Sepharose. It was essential, however, to exclude artifacts of an S-S-linked monomer/dimer type like observed with bacterial and mammalian thioredoxins [24]. Such interconversion does not occur among the algal thioredoxins since column chromatography of the oxidized proteins in absence of dithiothreitol, or in reducing, dithiothreitol-containing buffers nowhere led to appearance of different protein peaks.

It is not possible to assemble a purification scheme for all thioredoxins of *Scenedesmus* before separation of the individual species. Table I summarizes the purification of the two predominant, equally active species I and II, assayed with the best suited (in terms of quantification) enzyme system, *E. coli* ribonucleotide reductase. Specific activities are arbitrarily defined and are only calculated for comparison. The data indicate an at least 450-fold purification of the main thioredoxin couple from algal extracts in 13% recovery.

Thioredoxins in chloroplast-free algae

Extracts from heterotrophically grown algae of the *S. obliquus* mutant C-2A′ [25] have been fractionated and assayed for thioredoxin activity in the same way as extracts from green wild type cells. Whereas the mutant cell extract completely lacked thioredoxin *f* [7] it did contain minor thioredoxin III in the eluate from DEAE cellulose as well as both species I and II after resolution on CM cellulose. These three thioredoxins were quantitatively and qualitatively identical whether isolated from wild type *Scenedesmus obliquus*, from the mutant, or from mutant cells after 24 h greening in the light. Wild type algae grown on glucose in the dark also showed the same thioredoxin profile. These results indicate that thioredoxins I–III are no chloroplastic proteins.

Properties

The molecular weight of the three pure algal thioredoxins, in the reduced form, has been established by electrophoresis on SDS-containing polyacrylamide gels (Fig. 4). Species I, II, and III all belong to the typical M_r = 12,000 category of thioredoxins. The homogeneity of the chromatographically separated proteins I and II has been verified by electrophoresis under native conditions (right pattern in Fig. 4) because their mixture would not have been resolved on SDS gels. Slightly different isoelectric points (I: 5.2; II: 5.4) were determined

Table I. Purification of the main thioredoxin isoproteins BI and BII from extracts of *Scenedesmus obliquus*. Thioredoxin activity is expressed as nmol cytidylate reduced per 30 min in thioredoxin-dependent *E. coli* ribonucleotide reductase assays; substrate reduction in absence of thioredoxin has been subtracted.

Purification step	Total protein [mg]	Total activity [nmol CDP]	Recovery [%]	Spec. activity [nmol CDP·mg^{-1}]
Cell-free homogenate	915	6690[a]	(< 100)[a]	7
Supernatant after streptomycin sulfate/acid precipitation	230	18720	100	81
Supernatant after heat denaturation	94	13440	72	143
DEAE cellulose chromatography, fractions 28–40 (Fig. 1)	12	9160	49	764
Sephadex G-50 chromatography, fractions 37–47	3.0	5340	29	1780
CM cellulose chromatography				
thioredoxin I	0.44	1260	13	3310
thioredoxin II	0.31	1210		

[a] Inaccurate figures due to inhibition of the test enzyme by crude cell extract components.

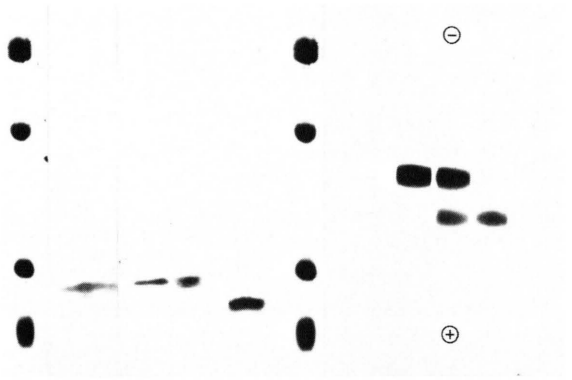

Fig. 4. Analysis of *S. obliquus* thioredoxins by polyacrylamide gel electrophoresis. Lanes 1–6: 15% gels in SDS-containing medium; lanes 7–9: 7.5% gels, under native conditions. – Lanes 1 and 6: Marker proteins; lane 2: thioredoxin III; lane 3: thioredoxin I; lane 4: thioredoxin II; lane 5: *E. coli* thioredoxin (M_r = 11,700). Lane 7: Thioredoxin I; lane 8: thioredoxin mixture before CM cellulose chromatography (I + II); lane 9: thioredoxin II. Marker proteins are (from above): Egg white albumin, M_r = 45,000; chymotrypsinogen, M_r = 25,000; cytochrome *c*, M_r = 12,500; and aprotinin, M_r = 6500.

Table II. Amino acid composition of thioredoxins I, II, and III from *S. obliquus*.

Amino acid	I	II	III
		[no. of residues]	
Ala	14	9	7
Arg	3	3	3
Asx	12	10	10
Cys[a]	2	2	2
Glx	12	11	14
Gly	11	12	11
His	1	2	2
Ile	3	3	3
Leu	7	9	6
Lys	6	7	6
Met	2	2	1
Phe	3	2	2
Pro	5	8	8
Ser	8	8	9
Thr	6	5	7
Trp[b]		not determined	
Tyr	2	2	3
Val	6	6	6
Total	103	101	100
M_r(calc.)[c]	12520	12360	11950

[a] Determined as S-carboxymethyl cysteine.
[b] The absorption and fluorescence spectra indicate presence of at least 1 tryptophan residue.
[c] Without tryptophan.

by isoelectric focussing and are in accord with the electrophoretic mobility.

The amino acid analysis of *Scenedesmus* thioredoxins I–III is presented in Table II. They contain two cysteine residues like all microbial thioredoxins analyzed so far. The composition of the three proteins is very similar; small differences in the number of acidic and basic amino acid residues may explain the different elution from anion and cation exchange columns. In general, the observed amino acid compositions do not match those of other plant (spinach [5], wheat [26]) thioredoxins but we note a certain similarity with the data reported for a cyanobacterial (*Anabaena* sp.) [13] and for yeast thioredoxin [27].

Other typical thioredoxin properties of the three *Scenedesmus* proteins include heat stability up to 90 °C without significant loss of enzyme stimulating activity, and fluorescence emission spectra with a broad maximum at 350–355 nm. In contrast to the spectral behaviour of *E. coli* thioredoxin [28] or to that of thioredoxin *f* [7], however, the fluorescence intensity of *Scenedesmus* thioredoxins I, II, and III remains unchanged in the oxidized and reduced states, respectively.

Activity in enzyme systems

Besides activation of the three test enzymes used during purification, the stimulatory effects of reduced algal thioredoxins I–III (*i.e.*, in presence of low dithiothreitol concentrations) have been determined in a number of other heterologous and homologous enzyme systems. Stimulation of cyanobacterial PAPS sulfotransferase by an unresolved mixture of I and II (= thioredoxin "B") had been noted earlier [3].

Most significant is the high activity of thioredoxins I and II with three different ribonucleotide reductases. Fig. 5 demonstrates their virtually identical reactivity as hydrogen donors of *E. coli* ribonucleotide reductase ($K_{m(app.)} = 5 \times 10^{-6}$ M). Deoxyadenosylcobalamin-dependent ribonucleotide reductase of the cyanobacterium *Anabaena* sp. will also efficiently accept algal thioredoxins (data not shown). Iron-requiring ribonucleotide reductase of *Scenedesmus obliquus* utilizes algal thioredoxins I and II even more specifically with an apparent K_m value of 0.1×10^{-6} M, compared with $K_{m(app.)} = 14 \times 10^{-6}$ M for *E. coli* thioredoxin [11]. These figures clearly indicate the functioning of plant thioredoxins in deoxyribonucleotide biosynthesis.

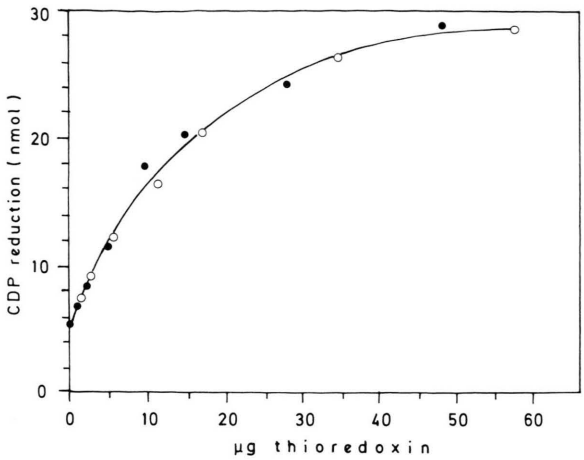

Fig. 5. Activation of *E. coli* ribonucleotide reductase by *S. obliquus* isothioredoxins. ●: Thioredoxin I; ○: thioredoxin II. Enzyme activity was measured in standard 30-min assays with CDP as substrate and at limiting (2 mM) dithiothreitol concentration.

Scenedesmus thioredoxins I and II react reasonably well with spinach chloroplast fructose-bis-phosphatase (*e.g.*, see Fig. 1). A complex behaviour was observed in the interaction between *Scenedesmus* thioredoxins and chloroplastic, light-activated NADP malate dehydrogenase (Fig. 6). Again the heterologous enzyme from spinach was stimulated more efficiently and with a simple saturation curve. The enzyme isolated from *Scenedesmus* cultures, besides being activated by algal thioredoxin *f* [7], reacted rapidly with algal thioredoxins I or II but only to a limited degree and in a narrow concentration range; elevated thioredoxin concentrations produced negligible stimulation. *E. coli* thioredoxin behaved like the algal proteins. For reasons discussed below, this *in vitro* reactivity appears inadequate to typify species I and II as *"m"* thioredoxins.

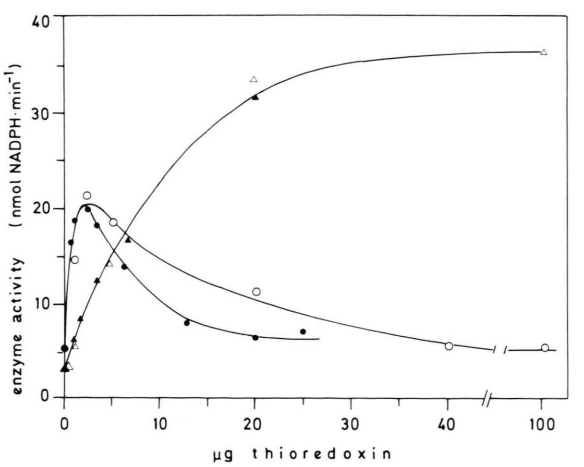

Fig. 6. Activation of chloroplast NADP malate dehydrogenases by thioredoxins. Circles: *Scenedesmus obliquus* enzyme in the presence of *S. obliquus* thioredoxin I or II (●) and *E. coli* thioredoxin (○). Triangles: Spinach enzyme in the presence of *S. obliquus* thioredoxins I or II (▲) and *E. coli* thioredoxin (△). Activation assays were incubated for 30 min.

The specific activity of thioredoxin III was only 10–35% of that of thioredoxins I and II in all the described systems; however it was always clearly above that of thioredoxin-free controls. The material has been tested for glutaredoxin activity [29] in ribonucleotide reductase assays coupled to glutathione, glutathione reductase, and NADPH in absence of dithiothreitol. Compared with an authentic glutaredoxin *(E. coli)* sample, thioredoxin III produced only 6% of the control activity which was not considered sufficient evidence for an algal glutaredoxin.

As mentioned previously [7], we have not been able to detect a thioredoxin reductase in extracts of *Scenedesmus obliquus*. When the oxidized algal thioredoxins (*i.e.*, in aerobic solutions without dithiothreitol) were tested with spinach chloroplast ferredoxin-thioredoxin reductase [23], protein *f* was as good a substrate as spinach thioredoxin *f* and *m*, and *Scenedesmus* thioredoxins I, II, and *E. coli* thioredoxin were still reduced at 75% the rate of the chloroplast proteins. In contrast to the specific NADPH thioredoxin reductases, which will not accept heterologous thioredoxins as substrates, the ferredoxin-dependent enzyme is thus a very unspecific one.

Discussion

A complete pattern of thioredoxins in the unicellular green alga *Scenedesmus obliquus* has been established in this and in a preceding communication [7]. The algae contain one large chloroplast thioredoxin of the *f* type and three proteins of regular size, M_r = 12,000, not located in the chloroplast. Thioredoxins I and II can certainly be termed cytoplasmic proteins on a functional basis because they show high activity in the cytoplasmic ribonucleotide reductase reaction. The nature of thioredoxin III, with uncharacteristicly

low enzyme activity, remains unclear. It is not known whether other cell organelles (*e.g.*, the mitochondria) contain thioredoxins and have to be considered as possible source of such minor species.

The thioredoxin content of a closely related green alga, *Chlorella pyrenoidosa,* has been analyzed using PAPS sulfotransferase of *E. coli* as the only indicator enzyme and two fractions, CP I, CP II of apparent molecular weight 14,000 were found [30]. On closer inspection we feel that CP II, a small fraction of low activity, could correspond to *Scenedesmus* thioredoxin III whereas CP I, which is resolved into two bands on native electrophoresis gels, could be a mixture of two proteins like *Scenedesmus* thioredoxins I and II. Thus, both green algae probably have the same set of three polypeptides.

Cyanobacteria have one regular thioredoxin and one large thioredoxin *f* [13]. The green algae differ from the prokaryotes not in the thioredoxin engaged in light-dependent functions but in the greater number of regular-size polypeptides. It is interesting to note that non-green tissues of higher plants like wheat and soy bean seeds, etiolated seedlings, and roots also contain three regular-size thioredoxins, usually grouped in two predominant, very similar proteins and one minor species like in *Scenedesmus* [10, 26]. Yeast cells also contain two thioredoxins I and II [27]. Thioredoxin duality or triplicity may actually be more common among eukaryotes.

The multitude of thioredoxins in non-photosynthetic plant cells raises the question of functional specialization in particular as the number of known thioredoxin functions in bacteria and animal cells is still growing [8]. At present we can only identify one role for thioredoxins I and II. Characterization of these two proteins and of ribonucleoside diphosphate reductase in *Scenedesmus obliquus* [11] has, for the first time, provided a complete *in vitro* system for plant deoxyribonucleotide biosynthesis. Such attempts had remained unsuccessful in higher plant extracts. The alternative glutaredoxin system, present in *E. coli* and in animal cells [29] could not be found.

Of course we do not claim that the cytoplasmic algal thioredoxins function exclusively in DNA precursor formation; general protein disulfide reductase or methionine sulfoxide reductase may also be thioredoxin-linked but are not easily measured. Likewise it is impossible to assign a physiological role to minor thioredoxin III. One would expect a rather specific one, probably not yet tested at all, because the protein differs strongly in activity from the two others despite remarkable similarity in amino acid composition.

Thioredoxins I and II react with chloroplast NADP malate dehydrogenase *in vitro*. It is not likely that this is of biochemical significance because of the different intracellular localization and the narrow concentration range (of unexplained nature) in which the activating effect manifests itself in a homologous protein system (Fig. 6). The observed *in vitro* activation will simply reflect the fairly unspecific reaction of these plant enzymes with thiols and thioredoxins, including liver thioredoxin [31, 32]. Therefore, the algal thioredoxins should not be classified as the *m* type. These data are another example for the notion that heterologous enzymes and thioredoxins in combination are frequently more active and less specific than homologous systems, and that caution is required in deducing physiological relationships from too small a number of thioredoxin assays. Where available, the use of homologous enzymes is recommended for unambiguous characterization of plant thioredoxins [33].

We conclude that the large number of thioredoxins in plants originates from the combination of several chloroplast proteins (probably derived from large cyanobacterial and algal precursor forms [7]) plus several cytoplasmic thioredoxins which are basically of the same kind as the thioredoxins of all other organisms. The *in vitro* exchangeability of most of these compounds, of as yet unknown sequence homologies and family relations, makes the assignment of individual functions *in vivo* a continuing challenge.

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[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] W. Wagner, H. Follmann, and A. Schmidt, Z. Naturforsch. **33c,** 517–520 (1978).
[4] B. B. Buchanan, Ann. Rev. Plant Physiol. **31,** 341–374 (1980).
[5] P. Schürmann, K. Maeda, and A. Tsugita, Eur. J. Biochem. **116,** 37–45 (1981).
[6] J. Buc, M. Riviere, B. Gontero, P. Sauve, J. C. Meunier, and J. Ricard, Eur. J. Biochem. **140,** 199–202 (1984).
[7] P. Langlotz, W. Wagner, and H. Follmann, Z. Naturforsch. **41c,** 275–283 (1986).
[8] A. Holmgren, Annu. Rev. Biochem. **54,** 237–271 (1985).
[9] N. A. Crawford, B. C. Yee, A. N. Nishizawa, and B. B. Buchanan, FEBS Lett. **104,** 141–145 (1979).
[10] A. Berstermann, K. Vogt, and H. Follmann, Eur. J. Biochem. **131,** 339–344 (1983).
[11] R. Hofmann, W. Feller, M. Pries, and H. Follmann, Biochim. Biophys. Acta **832,** 98–112 (1985).
[12] A. Schmidt, Arch. Microbiol. **127,** 259–265 (1980).
[13] F. K. Gleason and A. Holmgren, J. Biol. Chem. **256,** 8306–8309 (1981); M. M. Whittaker and F. K. Gleason, *ibid.* **259,** 14088–14093 (1984).
[14] N. I. Bishop and H. Senger, Methods Enzymol. **23,** 53–66 (1971).
[15] T. C. Laurent, E. C. Moore, and P. Reichard, J. Biol. Chem. **239,** 3436–3444 (1964).
[16] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and F. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[17] A. M. Crestfield, S. Moore, and W. H. Stein, J. Biol. Chem. **238,** 622–627 (1963).
[18] W. Wagner and H. Follmann, Biochem. Biophys. Res. Commun. **77,** 1044–1051 (1977).
[19] B. M. Sjöberg and A. Holmgren, Biochim. Biophys. Acta **315,** 176–180 (1973).
[20] N. C. Brown, Z. N. Canellakis, B. Lundin, P. Reichard, and L. Thelander, Eur. J. Biochem. **9,** 561–573 (1969).
[21] F. K. Gleason and T. D. Frick, J. Biol. Chem. **255,** 7728–7733 (1980).
[22] B. B. Buchanan, P. Schürmann, and P. K. Kalberer, J. Biol. Chem. **246,** 5952–5959 (1971).
[23] A. de la Torre, C. Lara, R. A. Wolosiuk, and B. B. Buchanan, FEBS Lett. **107,** 141–145 (1979).
[24] E. C. Herrmann and E. C. Moore, J. Biol. Chem. **248,** 1219–1223 (1973).
[25] H. Senger and N. I. Bishop, Plant Cell Physiol. **13,** 633–649 (1972).
[26] K. Vogt and H. Follmann, Biochim. Biophys. Acta **873,** 415–418 (1986).
[27] P. G. Porqué, A. Baldesten, and P. Reichard, J. Biol. Chem. **245,** 2363–2370 (1970).
[28] A. Holmgren, J. Biol. Chem. **247,** 1992–1998 (1972).
[29] A. Holmgren, J. Biol. Chem. **254,** 3664–3671 (1979).
[30] M. L. S. Tsang, Plant Physiol. **68,** 1098–1104 (1981).
[31] A. Holmgren, B. B. Buchanan, and R. A. Wolosiuk, FEBS Lett. **82,** 351–354 (1977).
[32] R. Scheibe, K. Fickenscher, and A. R. Ashton, Biochim. Biophys. Acta **870,** 191–197 (1986).
[33] I. Häberlein, G. Schimpff-Weiland, and H. Follmann, Biochem. Biophys. Res. Commun. **127,** 401–406 (1985).