A THIOREDOXIN FROM GREEN ALGAE

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SUMMARY. Deoxyribotide biosynthesis in plant cells includes thioredoxin for hydrogen transfer. A heat-stable protein of mol. weight 12,600 has been purified from the algae, Scenedesmus obliquus which in its reduced form stimulates CDP reduction by Scenedesmus as well as by Escherichia coli ribonucleotide reductase. It is not a substrate for E. coli thioredoxin reductase. The algal thioredoxin is similar to bacterial or mammalian thioredoxins but entirely different from the system described in Euglena. NADPH appears to be the ultimate reductant but thioredoxin reductase could not as yet be found in the algae extracts.

INTRODUCTION

The hydrogen donor system required for enzymatic reduction of ribonucleotides to deoxyribonucleotides has been found to consist of a small protein, thioredoxin, and a NADPH-dependent enzyme, thioredoxin reductase, in all microbial and mammalian cells studied so far. Thioredoxins isolated from E. coli, yeast, liver, or tumor cells (1-6) have molecular weights of about 12,000 and at their active center possess two cysteine residues; in this form they are substrates of ribonucleotide reductase (a), and the reduced form is then regenerated by thioredoxin reductase (b):

$$\text{thioredoxin(SH)}_2 + \text{NDP} \xrightarrow{a} \text{dNDP} + \text{thioredoxin-S}_2$$

$$\text{thioredoxin-S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{b} \text{thioredoxin(SH)}_2 + \text{NADP}^+ .$$

More recently, another hydrogen pathway utilizing glutathione and a novel protein called glutaredoxin was demonstrated in

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E.coli (7). At the same time, functions different from DNA precursor biosynthesis were ascribed to thioredoxins, e.g. the capacity of E.coli or calf liver thioredoxin to reduce protein disulfide bonds in insulin or human gonadotropin (8), or a regulatory role for photosynthesis in spinach chloroplasts (9). It is at present difficult to assess whether thioredoxin is an unspecific cellular reductant, or where it is a specific one. Further studies on the distribution of these proteins are clearly needed.

Thioredoxin could not as yet be demonstrated as the hydrogen source of ribonucleotide reduction in plants.The latter reaction has been assayed with unphysiologic reductants such as dithiothreitol in wheat and broad bean (10,11) but the search for an endogenous thioredoxin remained unsuccessful; if present, it will escape detection due to the existence of highly active, unspecific protein disulfide reductases (12, 13). Cells of the algae, Euglena gracilis were reported to contain a thioredoxin-like activity of highly unusual molecular size (240,000 daltons) (14). This system may not be representative for the plant kingdom because the coenzyme B_{12}-requiring ribonucleotide reductase of Euglena also differs strongly from other plant enzymes (15,16).

We have previously characterized a ribonucleoside diphosphate reductase in the green algae, Scenedesmus obliquus and Chlorella pyrenoidosa (16). A protein fraction has now been purified from the same source which, on the basis of its properties and its ability to promote ribonucleotide reduction is thioredoxin.

MATERIALS AND METHODS

Thioredoxin and thioredoxin reductase of E.coli were pre-

pared as described (1,2). Large scale preparation of ribonucleotide reductase from E.coli B3 (ATCC 23851) was performed by the published procedure (17) up to DEAE-cellulose chromatography. Solutions of radioactive [5-^{3}H]cytidine diphosphate (Amersham, England; spec.activity, 19 Ci/mmole) were freed from ethanol by evaporation prior to use.

Axenic cultures of Scenedesmus obliquus (strain D3) were grown under continuous light in inorganic, aerated media (3 % CO_2) at 25° up to a density of 5 µl packed cell volume per ml (18). Cells were harvested by centrifugation at 2,500 rpm. All subsequent operations were carried out at 0-5°C. The buffers contained 5 mM mercaptoethanol; pH values were adjusted at room temperature before thiol addition.

Preparation of thioredoxin: Cells were washed with Tris-HCl buffer (pH 7.5, 0.05 M) and broken in a Bühler-Vibrogen cell mill with glass beads of 0.7 mm diameter. The beads were removed by filtration through a glass frit, and cell debris by centrifugation for 30 min at 20,000 x g.

To the extract was added 4% streptomycin sulphate solution to a final concentration of 0.8 %. After 1 hr the precipitate was removed and the supernatant was fractionated with ammonium sulphate. The precipitate formed at 40% saturation (231 mg salt added per ml at 0°) after 2 hrs was discarded; thioredoxin was precipitated at 80% saturation by further addition of 271 mg salt/ml and was collected by centrifugation. The pellet was redissolved and dialysed overnight against phosphate buffer (pH 7.3, 0.02 M). The solution was then heated to 60° for 5 min under vigorous stirring and after rapid cooling the precipitate was removed. The supernatant was made 5 mM in dithiothreitol and kept for 1 hr at room temperature to obtain fully reduced protein (6).

The solution was passed through a DEAE-cellulose column (pretreated with 1 M phosphate, pH 7.3, and equilibrated with water containing 5 mM mercaptoethanol) and thioredoxin was eluted with 0.02 M phosphate; approximately 80 % of protein and pigments remained bound at this low ionic strength. Active fractions were precipitated with ammonium sulphate, redissolved, reduced in 5 mM dithiothreitol, and chromatographed over Sephadex G-75 in Tris-HCl (pH 7.5) (fig.1).

Thioredoxin assay: Assay mixtures with E.coli ribonucleotide reductase contained, in a final volume of 0.3 ml: 0.8 mg enzyme; 10 µCi [^{3}H]CDP (diluted to 0.23 Ci/mmole spec.activity); 2.33 mM dithiothreitol; 15 mM Mg-acetate; 0.8 mM EDTA; and 0.115 mM dTTP as effector. Thioredoxin-containing fractions in Tris buffer (pH 7.5) were added up to 0.15 ml volume. The mixtures were incubated at 30° for 30 min and after addition of 0.25 µmoles CMP and dCMP the reaction was terminated with 1 M perchloric acid. Sample nucleotides were hydrolysed to the monophosphates and separated on PEI cellulose thin layer plates developed in 1 M LiCl satd with H_3BO_3/ethanol 1:1 (19). Radioactivity was determined on a thin layer scanner equipped with integrator (BF model LB 2723).

Assay mixtures with partially purified Scenedesmus ribonucleotide reductase (16) contained: 0.6 mg protein; 10 µCi [^{3}H]CDP (spec.activity, 19 Ci/mmole); 2 mM dithio-

threitol; 5 mM Mg-acetate; 0.06 mM Fe^{++}; 8 mM KF; and 3.5 mM ATP as effector. After 1 hr at 30° the mixtures were analysed as above.

RESULTS AND DISCUSSION

The detection of unknown thioredoxins, which themselves possess no enzymic activity, is difficult when the endogenous enzymes, ribonucleotide reductase and thioredoxin reductase are not available. In seedlings of higher plants as well as in extracts of algae the NADPH-coupled, spectrophotometric assay of 5,5'-dithiobis(2-nitrobenzoate) reduction (1) proved too unspecific to identify protein fractions of thioredoxin activity. We therefore chose to test for thioredoxin in Scenedesmus obliquus by measuring the dithiol-dependent CDP reduction catalysed by ribonucleotide reductase purified from E.coli (17). The latter enzyme appears to lack species specificity with respect to the origin of its reductants; when assayed in the presence of low concentrations of dithiothreitol it is stimulated by addition of E. coli as well as yeast, liver, or tumor thioredoxin. It was found that the same is true for a thioredoxin fraction prepared from the eucaryotic algae (Table 1). Partially purified ribonucleotide reductase from synchronous cultures of Scenedesmus, although only available at lower specific activity, is also stimulated significantly in vitro.

Table 1 summarizes purification steps for the stimulatory fraction. Following streptomycin treatment and ammonium sulphate fractionation of the crude extract the preparation was subjected to heat denaturation at 60°; heat stability is a typical property of thioredoxins. In contrast to other thioredoxins the algal protein was not bound to DEAE-cellulose at

Table 1. Stimulation of ribonucleotide reductase-catalysed CDP reduction by Scenedesmus thioredoxin *

Purification step	E.coli enzyme		S.obliquus enzyme	
	%	% / mg	%	% / mg
crude extract	5	2		
$(NH_4)_2SO_4$ fraction	34	3	0	0
60° supernatant	87	38	144	63
DEAE-cellulose	79	179	56	127
Sephadex G-75	32	1066	22	733

* The activity of thioredoxin is expressed as % stimulation of reductase action over controls (-thioredoxin) in the standard assay, and as % stimulation/mg protein (specific stimulation), respectively. Absolute values of unstimulated CDP reduction: E.coli enzyme, 3.2 nmoles/mg/hr; Scenedesmus enzyme, 5 pmoles/mg/hr.

low ionic strength; nevertheless this column chromatography provided a satisfactory separation step. Subsequent gel filtration (Figure 1) yielded highly active material about 1,000-fold enriched over the crude extract. When assayed with Scenedesmus enzyme this purest fraction supported CDP reduction in the absence of metal ions which do not appear to be essential for algal ribonucleotide reductase (W.Feller, unpublished). A molecular weight of 12,600 daltons was estimated from gel filtration experiments. This is almost identical with the size of most other known thioredoxins, but entirely different from the large protein described to function like thioredoxin in Euglena (14). The chloroplast factor later identified as thioredoxin shows an approximate molecular weight of 20,000 (21). Scenedesmus thioredoxin is stable at pH values above 4.5. It is rapidly inactivated (oxidized) in the absence of thiols but may be regenerated by incubation with dithiothreitol (5 mM).

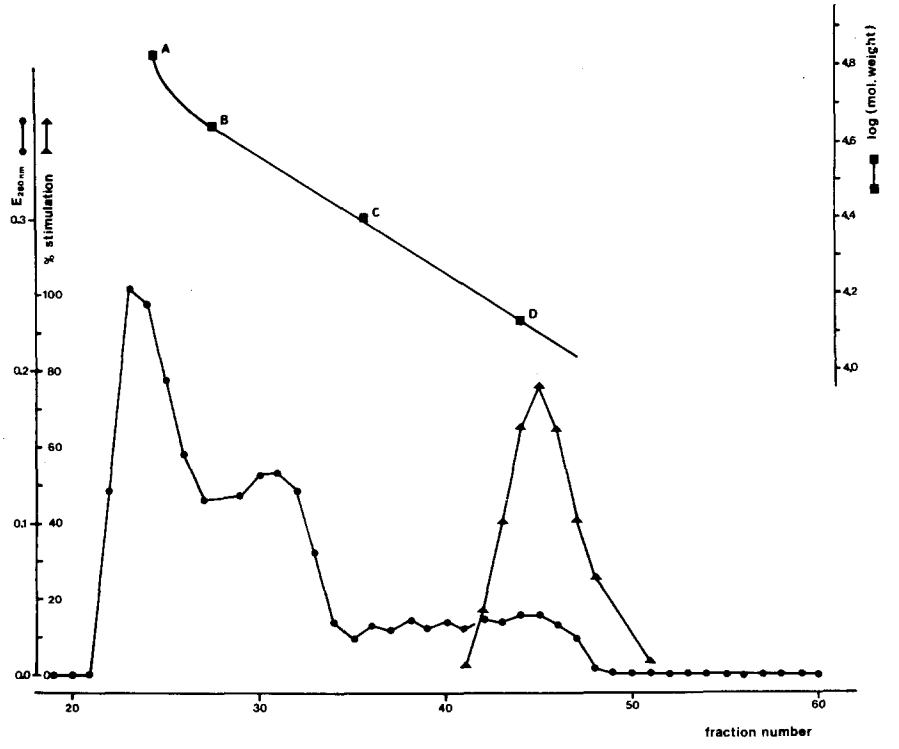

Figure 1. Gel filtration of Scenedesmus thioredoxin on Sephadex G-75 in Tris-HCl (0.05 M, pH 7.5, + 5 mM mercaptoethanol). Column size, 53 x 2 cm; flow rate, 8.6 ml/hr, fraction volume, 2.2 ml. Marker proteins for molecular weight determination: A, serum albumin; B, ovalbumin; C, chymotrypsinogen; D, cytochrome c.

Active Scenedesmus thioredoxin alone cannot support CDP reduction in the absence of a ribonucleotide reductase. Likewise it is not a substrate of E.coli thioredoxin reductase, in agreement with previous observations on the species specificity of that enzyme. It was not as yet possible to isolate endogenous thioredoxin reductase from batch cultures of the green algae. Passage of ammonium sulphate fractions over a column of 2',5'-ADP-Sepharose which efficiently retains the E.coli and liver enzymes (20) permitted purification of algal glutathione reductase, but not thioredoxin reductase.

It is possible that the plant enzyme was not bound to this affinity adsorbent due to major structure dissimilarities, or that it can only be found in synchronized cells like the ribonucleotide reductase (16). A ferredoxin-linked thioredoxin reductase like the one described for chloroplasts (9) is less likely because CDP reduction in Scenedesmus extracts is initially stimulated severalfold by NADPH (1 mM), but not after heat treatment of protein fractions. An unusual protein structure of Scenedesmus thioredoxin reductase would parallel that of the organism's ribonucleotide reductase which is much smaller than all other diphosphate reductases (mol. weight, 85,000) and lacks metal ion requirement (W.Feller, unpublished results).

These results confirm thioredoxin function for deoxyribotide biosynthesis in green algae. Nothing can be said about cytoplasmatic or chloroplast origin of the reducing protein but the reductase of which it is a substrate clearly functions in nuclear, and not in chloroplast DNA biosynthesis (16). Further studies on the specificity and the amino acid composition of Scenedesmus thioredoxin are in progress.

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