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Thioredoxin-like Activity of Thylakoid Membranes

THIOREDOXIN CATALYZING THE REDUCTIVE INACTIVATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE OCCURS IN BOTH SOLUBLE AND MEMBRANE-BOUND FORM[1]

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ABSTRACT

The inactivation of pea leaf chloroplast glucose-6-phosphate dehydrogenase by dithiothreitol can be catalyzed by thioredoxin-like molecules that are present in chloroplasts. This thioredoxin activity occurs predominantly as a soluble species, but washed thylakoid membranes also exhibit some thioredoxin-like activity. The membrane-associated thioredoxin can be extracted by treatment with the detergent Triton X-100. The solubilized thioredoxin appears to have a molecular size similar to that of the soluble thioredoxin which catalyzes the same reaction. The thylakoid-bound activity constitutes only about 5% of the total chloroplast thioredoxin activity. The thioredoxin occurring in the membrane fraction cannot, however, be ascribed to the trapping of stroma since less than 0.1% of three stromal marker enzymes are found in the same thylakoid extract.

Thioredoxin is a polypeptide containing 2 cysteine residues which can undergo reversible oxidation and reduction. Thioredoxins which have been characterized from bacteria, yeast, and animals are soluble proteins of about 100 residues which have defined roles in ribonucleotide reduction and sulfate reduction (*e.g.* see ref. 10, 13).

Several reports of thioredoxin-like activity in plants have appeared. Asahi and Masaki (4) described a photoreducible polypeptide present in chloroplasts which participates in light-dependent sulfate reduction. Thioredoxins which can participate in ribonucleotide reduction were reported by Wagner and Follman (19). Buchanan and co-workers described a multiplicity of soluble thioredoxins in both chloroplasts and cytoplasm (20). The chloroplast thioredoxins have been proposed as mediators in the light-dependent regulation of chloroplast enzyme activity (6).

Most workers have assumed that all thioredoxin activity is soluble, although both Kagawa and Hatch (12) and Scheibe and Beck (17) demonstrated some thioredoxin-like activity in chloroplast membranes. Whether this activity was an entrapped soluble form or a specific membrane-bound form was not shown. Scheibe and Beck (17) reported that the membrane thioredoxin-like activity could be readily removed in dilute buffer. Here, we present evidence that chloroplast membranes possess a thioredoxin activity which is not removed by extensive washing but which is solubilized by the detergent Triton X-100. Furthermore, we show that the inactivation of chloroplast G6PDH[4] by DTT (2, 11) can be catalyzed by thioredoxin.

MATERIALS AND METHODS

Pea (*Pisum sativum* L., var. Little Marvel) plants were grown in Vermiculite in a greenhouse. Chloroplasts were prepared as described in Cockburn *et al.* (7), but in the absence of sodium isoascorbate, and washed once in 0.33 M sorbitol containing 50 mM Hepes (K^+), 2 mM $MgCl_2$, and 2 mM EDTA (K^+) (pH 7.2).

Enzyme Assays. The buffer present in all enzyme assays was 25 mM Tricine, 1 mM EDTA adjusted to pH 8.3 with KOH. All enzyme activities were measured at room temperature (about 20 C) by following the A change of the 1-ml reaction mixture using either a Cary 219 or a Gilford 2400 recording spectrophotometer.

Components of individual enzyme assays were as follows: G6PDH (EC 1.1.1.49), 1 mM glucose-6-P and 0.25 mM $NADP^+$; glutathione reductase (EC 1.6.4.2.), 1 mM oxidized glutathione and 0.1 mM NADPH; $NADP^+$ malate dehydrogenase (EC 1.1.1.82.), 50 mM L-malate (K^+) (pH 8.3) and 0.25 mM $NADP^+$; ferredoxin-NADP reductase (EC 1.18.1.2), 0.05 mM dichlorophenolindophenol and 0.1 mM NADPH.

Ferredoxin-NADP reductase activity was monitored by following the reduction of dichlorophenolindophenol at 600 nm, whereas all other enzyme activities were monitored by following the oxidation or reduction of pyridine nucleotide at 340 nm.

Chl and Protein Determination. Chl was estimated by its A at 652 nm in 80% (v/v) acetone: $E_{652}^{1mg/ml} = 28.99$ (5). Protein was determined by the procedure of Scopes (18).

Mol Wt Estimation. A Sephacryl S-200 column (1.1 $cm^2 \times 56$ cm) was calibrated by measuring the elution volumes of the proteins Cyt *c* (mol wt 12,400), ribonuclease A (13,700), soybean trypsin inhibitor (21,500) ovalbumin (45,000), phosphoglycerate kinase (48,000), lactate dehydrogenase (140,000), whereas thyroglobulin (mol wt 670,000) was used as a void volume marker.

Assay of Thioredoxin Activity. Thioredoxin was determined by its ability to enhance the rate of inactivation of chloroplast G6PDH by DTT.

The reaction mixture (25 μl, final volume) contained 10 μl partially purified chloroplast G6PDH (10^{-3} I units), 10 μl sample, and 5 μl 25 mM DTT, all in 50 mM Hepes, 1 mM EDTA, 2 mM $MgCl_2$, and 10 mM KCl (pH 7.4) (Hepes-KCl buffer). The mixture was incubated for 15 min at 25 C, and then inactivation was stopped by dilution into the G6PDH assay solution (1 ml, final volume). The control assay (to measure thioredoxin-independent inactivation) contained all components except thioredoxin.

The name thioredoxin was originally used to specify the hydro-

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[4] Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; LEM, light-effect mediator.

gen donor in the bacterial ribonucleotide reduction system (12). Subsequently, it was demonstrated that peptides isolated from plants which could activate various enzymes (20) would, like thioredoxin from *Escherichia coli*, stimulate CDP reduction by ribonucleotide reductase, although at only 1% of the rate of *E. coli* thioredoxin (9). For convenience, we have used the term thioredoxin for the species described in this report because of their similarity to the factors described and named thioredoxin by Buchanan and co-workers (20). This designation should be regarded as tentative since we do not know if this factor will function in the ribonucleotide reduction system.

Preparation of G6PDH. Chloroplasts, prepared as described above, were lysed in distilled H_2O (10 volumes) and the suspension was then adjusted to the proper concentration of the Hepes-KCl buffer with concentrated buffer. The broken chloroplast suspension was centrifuged (27,000*g* for 15 min) and the membrane pellet was discarded. The supernatant was brought to 45% saturation with respect to ammonium sulfate (2.06 M). The precipitate, containing the chloroplast G6PDH, was redissolved in Hepes-KCl buffer and fractionated on a Sephacryl S-200 column (5 $cm^2 \times 40$ cm) equilibrated with Hepes-KCl buffer. G6PDH eluted as a single peak near the void volume of the column, well resolved from any thioredoxin activity. The fractions containing G6PDH were pooled, divided into small aliquots, and frozen at −20 C until needed. The specific activity of this G6PDH was about 0.4 μmol NADPH formed/min·mg protein. Purification was 4-fold.

Extraction of Chloroplast Membranes. The chloroplast fraction was lysed by resuspension in distilled H_2O and then adjusted to standard Hepes-KCl buffer concentration by addition of concentrated buffer. The thylakoid fraction (21 mg Chl) was sedimented (27,000*g* for 10 min) and then washed twice with 250 ml of the same buffer.

The thylakoid membranes (2.5 mg Chl/ml) were extracted for 24 h at 4 C with 50 mM Tris (Cl^-) 200 mM KCl, and 1% (v/v) Triton X-100 (pH 9.0) (8) and then centrifuged at 50,000*g* for 60 min. A 2-ml aliquot of the supernatant solution was fractionated at 4 C on a Sephacryl S-200 column (1.1 $cm^2 \times 54$ cm) equilibrated with the Hepes-KCl buffer containing 1% (v/v) Triton X-100. Fractions of 0.96 ml (35 drops) were collected.

The stromal extract obtained from the lysed chloroplasts was fractioned by addition of ammonium sulphate to 70% saturation (3.57 M). The pellet was dissolved by overnight dialysis against Hepes-HCl buffer. The nondiffusible solution then was adjusted to 1% with respect to Triton X-100 and a 2-ml aliquot was fractionated on a Sephacryl S-200 column equilibrated with Hepes-KCl buffer containing 1% (v/v) Triton X-100. Fractions of 0.96 ml were collected.

Chemicals. Triton X-100, Sephacryl S-200, and all biochemicals were obtained from Sigma Chemical Co. Other chemicals were analytical reagent grade. Pea seeds were obtained from Northrup and King, Chicago.

RESULTS

Thioredoxin Activity of Chloroplasts. The presence of thioredoxin activity in the stromal fraction of lysed chloroplasts could be readily demonstrated. However, assay of the membrane fraction presented difficulties which tended to obscure thioredoxin activity. In the presence of membranes, a relatively high "inactivation" of G6PDH occurred in the absence of DTT. Nevertheless, there did appear to be some thioredoxin activity associated with the thylakoid membrane which survived prolonged washing.

Extraction and Chromatography of Membrane-associated Thioredoxin. Thioredoxin activity could be solubilized from thylakoid membranes with Triton X-100 using the procedure that Golbeck *et al.* (8) used for extracting PS I particles. Assay of thioredoxin activity in the insoluble residue is not possible because of the high nonspecific inactivation of G6PDH; thus, it was not possible to estimate the proportion of thioredoxin extracted. After the thioredoxin activity was solubilized, further purification was accomplished by gel filtration on a detergent-equilibrated Sephacryl S-200 column (Fig. 1). A single peak of thioredoxin activity eluted from the column as a low mol wt species. By reference to proteins of known mol wt, the thioredoxin was estimated to have a mol wt of 13,000 (the thioredoxin elutes between Cyt *c* and ribonuclease A) (Fig. 2). The mol wt of the spinach stromal thioredoxins has been estimated to be 9,000 and 16,000 (20). The thioredoxin was resolved from the higher mol wt Chl-containing peak which corresponds to the PS I complex of Golbeck *et al.* (8) and Lien and San Pietro (14). The gel filtration profile (Fig. 1) contained no detectable G6PDH (which would interfere with the thioredoxin assay).

Inactivation of G6PDH by DTT and Membrane-associated Thioredoxin. G6PDH from chloroplasts is inactivated in a time-dependent process by DTT (Fig. 3). Addition of thioredoxin extracted from membranes resulted in a 3-fold enhancement in the rate of inactivation of the G6PDH (Fig. 3).

Gel Filtration of Stromal Thioredoxin. A stromal fraction, concentrated by precipitation with ammonium sulfate, was fractionated on Sephacryl S-200 under identical conditions to those

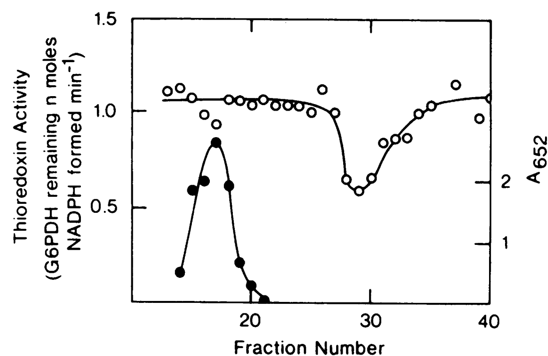

FIG. 1. Sephacryl S-200 gel filtration profile of detergent-solubilized thylakoid membranes. A 2-ml aliquot of detergent-solubilized thylakoid membranes (equivalent to 5 mg Chl) was subjected to gel filtration on a Sephacryl S-200 column (1.1 $cm^2 \times 54$ cm) equilibrated with 1 % Triton X-100. Fractions of 35 drops (0.96 ml) were collected. (●): Chl, A_{652}. Thioredoxin was detected by its ability to hasten the inactivation of G6PDH by DTT. The thioredoxin profile is displayed as the G6PDH activity remaining after the incubation with DTT and the respective fraction (○). Thus, the more thioredoxin was present, the lower was the G6PDH activity.

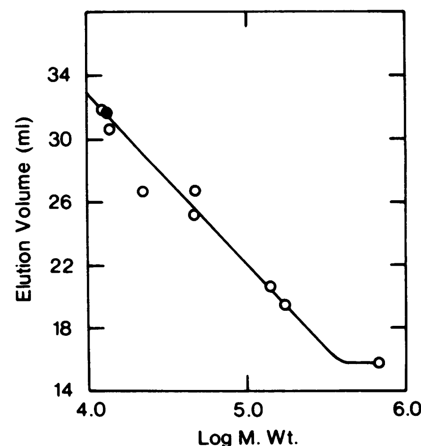

FIG. 2. Mol wt calibration curve of Sephacryl S-200 column. The column was calibrated with protein standards (○) as described under "Materials and Methods." (●): Membrane-associated thioredoxin.

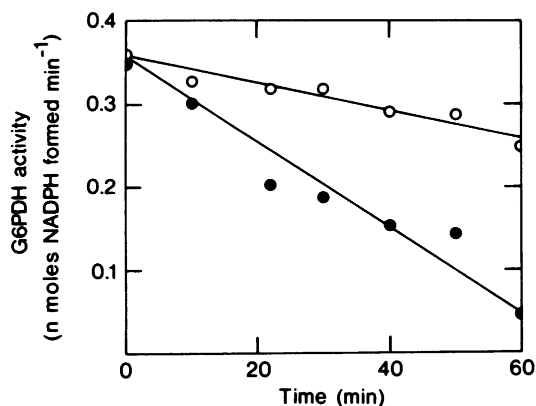

FIG. 3. Time course of inactivation of G6PDH catalyzed by DTT and membrane-associated thioredoxin. A thioredoxin assay mixture containing the solubilized, partially purified, membrane-associated thioredoxin (fraction 30 of Fig. 1) was incubated at 25 C and aliquots were removed and assayed for remaining G6PDH activity (●). The control reaction showing the DTT-dependent inactivation in the absence of any added thioredoxin is also shown (○).

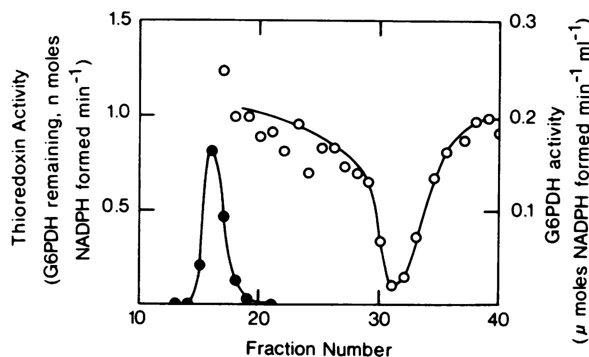

FIG. 4. Sephacryl S-200 gel filtration profile of chloroplast stromal (soluble) fraction. A 2-ml aliquot of stroma (concentrated by precipitation with 70% saturated ammonium sulfate and derived from 8.75 mg Chl) was subjected to gel filtration under conditions identical to those for the thylakoid extract shown in Fig. 1. The thioredoxin profile is illustrated by displaying G6PDH activity remaining after 5 min thioredoxin-catalyzed inactivation (○). The elution profile of the G6PDH present in the stroma (●) is also shown.

used for the solubilized fraction described above. A single peak of thioredoxin activity eluted as a low mol wt species (Fig. 4). This gel filtration profile, although conducted for comparative purposes in the presence of 1% (v/v) Triton X-100, is similar to profiles obtained in the total absence of detergent. The stromal G6PDH eluted near the void volume of the column and did not interfere with estimations of thioredoxin activity elsewhere on the column.

Estimation of Stromal Contamination of Thylakoid Membrane Fraction. The extent of stromal contamination of the thylakoid membrane fraction was estimated by examining the distribution of various enzymes between the soluble and thylakoid membrane-derived fraction. Table I shows that, for each of three stromal enzymes (G6PDH, glutathione reductase, and $NADP^+$ malate dehydrogenase), less than 0.1% of the total activity occurred in the thylakoid membrane-derived fraction. Ferredoxin-NADP reductase, on the other hand, which is partially membrane-bound, was more predominant in the thylakoid fraction. Thioredoxin activity occurred predominantly in the stroma, but a significant amount was present in the thylakoid fraction. The estimation of thioredoxin activity in crude extracts was complicated by the presence of endogenous G6PDH. Thus, the thioredoxin activity of fractions eluted from Sephacryl S-200 was used to estimate the

Table I. *Distribution of Enzymes between Stromal Fraction and Detergent-extracted Fraction*

Enzyme activities were determined from aliquots of the respective fractions taken prior to the Sephacryl S-200 chromatography step. The respective amounts of thioredoxin were estimated after the Sephacryl S-200 gel filtration steps as described under "Materials and Methods." The activity of G6PDH, glutathione reductase, and $NADP^+$ malate dehydrogenase in the stroma were 3.9, 19.5, and 16.0 μmol substrate disappearing/mg Chl·h, respectively. The activity of the ferredoxin-$NADP^+$ reductase in the stromal and thylakoid fraction was 8.1 and 30.2 A_{600} units disappearing/mg Chl·min, respectively.

Enzyme	Distribution	
	Stroma	Triton X-100 Extraction
	% total activity	
G6PDH	100	≤0.1
Glutathione reductase	100	≤0.1
$NADP^+$ malate dehydrogenase	100	≤0.1
Ferredoxin-$NADP^+$ reductase	21	79
Thioredoxin	95	5

relative distribution of activity in the stroma and thylakoid fractions.

DISCUSSION

Several laboratories have shown that low mol wt molecules (thioredoxins) can mediate the DTT-dependent activation of some chloroplast enzymes, including fructose-1,6-bisphosphatase (20) and $NADP^+$ malate dehydrogenase (12, 20). The present study extends the previous work by demonstrating that enzyme inactivation can also be mediated by thioredoxin. Furthermore, the present study demonstrates that a thioredoxin catalyzing the inactivation of G6PDH is associated with the thylakoid membrane. The thylakoid membrane fraction contains at least 50 times more thioredoxin activity than can be accounted for by simple stromal contamination. This suggests that a specific association occurs between the thioredoxin and the thylakoid membrane, but the nature of this interaction remains unclear. Whether the thioredoxin interacts directly with the lipid component of the membrane or through protein-protein interaction is not known. Thylakoid membranes washed in buffer containing 5 mM EDTA still contained membrane-associated thioredoxin activity. The identity, if any, of these thioredoxins catalyzing the inactivation of G6PDH with any of the thioredoxins characterized by others has not been determined.

The thylakoid extract can also catalyze the DTT-dependent activation of $NADP^+$ malate dehydrogenase (data not shown), suggesting that the solubilized thioredoxin may possess a broad specificity in its interaction with enzymes. This is in agreement with the findings of Wolusiuk *et al.* (20) who showed that thioredoxin f and thioredoxin m did not show absolute enzyme specificity with thioredoxin f activating $NADP^+$ malate dehydrogenase and, conversely, thioredoxin m activating fructose bisphosphatase.

Thioredoxin-like molecules in plants have been postulated to function in several metabolic processes including sulfate reduction (4), the light activation of various chloroplast enzymes (20), and ribonucleotide reduction (19). It seems that all thioredoxins described could function, to some degree, in each role. In the thioredoxin-dependent light activation scheme described by Buchanan and co-workers (6), no light activation can be demonstrated in the absence of added thioredoxin. This suggests that, in spinach at least, membrane thioredoxin does not participate in light activation. The membrane thioredoxin may function in the reduction of 5,5'-dithiobis(2-nitrobenzoic acid), by washed thyla-

koids described by Anderson *et al.* (3). Such speculation, of course, requires that the membrane-associated thioredoxin is reduced in the absence of the soluble components described by Wolusiuk *et al.* (20), *i.e.* ferredoxin and the ferredoxin-thioredoxin reductase. We have found that washed thylakoid membranes contain barely detectable levels of ferredoxin [less than 0.5% of stromal ferredoxin (A. R. Ashton and L. E. Anderson, unpublished data)], but we have not attempted to determine if any ferredoxin-thioredoxin reductase is bound to the membrane. The LEM, described by Anderson and Avron (1), is a membrane-associated factor which, when reduced by light-driven electron flow through PSI, can modulate chloroplast enzyme activity.

It is possible that chemically reduced LEM could also modulate chloroplast enzyme activity and, thus, exhibit thioredoxin-like activity. Since no detectable thioredoxin activity was associated with fractions containing the PS I particle, the low mol wt membrane-bound species that we have demonstrated may, in fact, be the LEM. The PS I particle can catalyze the light-dependent inactivation of G6PDH present in stroma (data not shown). Mve Akamba (16) has shown that PS I-enriched particles catalyze the light-dependent activation of $NADP^+$-malate dehydrogenase and NADP-glyceraldehyde-3-phosphate dehydrogenase present in whole stroma. Our experiments suggest that these PS I particles have retained some LEM function or that the stroma used as enzyme source can provide components that replace the extracted LEM. It seems less likely, then, that the extracted thioredoxin is the LEM. Our "thioredoxin" assay would be capable of detecting the LEM only if DTT can reduce the LEM more rapidly than it can reduce G6PDH. It is also conceivable that the membrane-bound thioredoxin may not function as an electron carrier at all but may constitute a subunit of a more complex enzyme as is the case for DNA polymerase of T7 phage (15).

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