OCCURRENCE OF CYTOPLASMIC *f*- AND *m*-TYPE THIOREDOXINS IN LEAVES

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1. Introduction

There is a growing body of evidence that thioredoxins (proteins that act as regulatory messengers in linking light to enzyme modulation in chloroplasts [1,2]) occur in multiple forms in photosynthetic cells [3–6]. Two types of thioredoxins are found in chloroplasts (thioredoxins *f* and *m*), and a third type is found outside chloroplasts, possibly in the cytoplasm (thioredoxin *c*) [6].

Chloroplast thioredoxins *f* and *m* are reduced photochemically by chloroplasts via ferredoxin and ferredoxin-thioredoxin reductase or, independently of light, in vitro by the nonphysiological sulfhydryl reagent dithiothreitol. When reduced, thioredoxin *f* activates specific chloroplast enzymes, including enzymes of the reductive pentose phosphate cycle (fructose-1,6-bisphosphatase (Fru-P_2ase), NADP-glyceraldehyde 3-phosphate dehydrogenase, phosphoribulokinase, sedoheptulose-1,7-bisphosphatase [6]) and an enzyme of secondary plant metabolism (phenylalanine ammonia lyase [7]). By contrast, thioredoxin *m* is specific in its activation of the single chloroplast enzyme, NADP-malate dehydrogenase (NADP-MDH [6]).

The function of cytoplasmic thioredoxin *c* is unknown. In our research, thioredoxin *c* resembled its chloroplast counterparts in promoting the activation of chloroplast enzymes. However, unlike chloroplast thioredoxins, thioredoxin *c* appeared to be nonspecific in that when reduced with dithiothreitol it effectively activated thioredoxin *m*- as well as thioredoxin *f*-linked enzymes.

Following these early studies on thioredoxin *c* specificity with several different partially purified preparations, we recently performed specificity studies using a single cytoplasmic thioredoxin preparation. We now present evidence that the thioredoxin *c* fraction in [6] contains two different thioredoxins: one that preferentially activates Fru-P_2ase (designated thioredoxin c_f) and one that preferentially activates NADP-MDH (designated thioredoxin c_m). Pending further evidence on intracellular location, the thioredoxins of the *c*-type are referred to as cytoplasmic thioredoxins.

Abbreviations: Fru-P_2ase, fructose 1,6-bisphosphatase; NADP-MDH, NADP-malate dehydrogenase; P_i, inorganic phosphate

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2. Methods

2.1. *Isolation of thioredoxins*

The procedures for the purification of thioredoxins *f* and *m* and of the thioredoxin *c* fraction were as in [6] except that 1.4 mM β-mercaptoethanol was added to all solutions. Each of the leaf thioredoxins was obtained from 5 kg frozen light-grown barley seedlings [8] or market spinach leaves by the procedure described for the isolation of the thioredoxin *c* fraction (through step IV, first DEAE-cellulose chromatography step [6]). At that point, the active fractions were combined in two lots: those that passed through the column directly (cytoplasmic thioredoxin *c* lot) and those that were retained on the column and eluted with the linear gradient (chloroplast thioredoxins *f* + *m* lot). Hereafter, the steps for further purification of each lot were as described for the purification of thioredoxins *f* and *m* from chloroplast extract: concentration by dialysis against solid sucrose (step

V), separation of the two thioredoxins in each lot (i.e., thioredoxin *f* from *m* and thioredoxin c_f from c_m) by Sephadex G-75 chromatography (step VI), and further purification of each of the four thioredoxins individually by hydroxyapatite chromatography (step VII). A separation of chloroplast thioredoxins *f* and *m* and of cytoplasmic thioredoxins c_f and c_m was achieved by this procedure.

For determination of the thioredoxins indigenous to chloroplasts, once-washed chloroplasts were isolated from 5 kg week 1 light-grown barley seedlings as described for spinach [6] except that the solutions used for osmotic breakage and all subsequent solutions were supplemented with 1.4 mM β-mercaptoethanol. The chloroplast extract so obtained was applied to a DEAE-cellulose column that was equilibrated beforehand with a solution of 30 mM Tris–HCl buffer (pH 7.9) and 1.4 mM β-mercaptoethanol (buffer A). The column was eluted sequentially with (i) 50 ml buffer A; (ii) 120 ml of a linear gradient (25–200 mM NaCl in buffer A); finally, (iii) with 50 ml buffer A containing 500 mM NaCl.

2.2. *Fru-P_2ase assay*

The activities of thioredoxins *f* and c_f were measured by the dithiothreitol Fru-P_2ase assay devised earlier [6]. Homogeneous spinach chloroplast Fru-P_2ase (16 μg) was preincubated for 10 min in test tubes containing (in 0.45 ml) Tris–HCl buffer (pH 7.9) 50 μmol; $MgSO_4$, 0.5 μmol; dithiothreitol, 2.5 μmol; and thioredoxin as needed. The reaction was initiated by adding 0.05 ml 60 mM sodium fructose 1,6-bisphosphate. The reaction was stopped after 15 min by the addition of 2 ml of the reagent used for P_i analysis [9].

2.3. *NADP-MDH assay*

The activities of thioredoxins *m* and c_m were measured by using the NADP-MDH/dithiothreitol assay devised [6]. Partially purified spinach chloroplast NADP-MDH (40 μg) was preincubated at room temperature in 0.2 ml of a solution containing: Tris–HCl buffer (pH 7.9), 20 μmol; dithiothreitol, 2 μmol; and thioredoxin as needed. After 5 min preincubation, the mixture was injected into a 1 cm cuvette that contained Tris–HCl buffer (pH 7.9) 100 μmol and NADPH, 0.25 μmol. The reaction was started by the immediate addition of 2.5 μmol oxaloacetic acid (final volume, 1.0 ml). The change in absorbance was measured with a Cary 14 spectrophotometer.

2.4. *Other methods*

Molecular weights of the barley leaf thioredoxins were determined by exclusion column chromatography on a calibrated 1.5 × 100 cm Sephadex G-75 column that was equilibrated and developed with buffer A [6]. Published procedures were used for the purification of spinach chloroplast Fru-P_2ase [10] and NADP-MDH [11]. Described methods were also used for the estimation of chlorophyll and protein and for polyacrylamide gel electrophoresis [6].

3. Results and discussion

3.1. *Identification of thioredoxins* c_f *and* c_m

The possibility that the described spinach leaf thioredoxin *c* fraction [6] contains two active components arose when it was noted that an aged preparation that had lost the capacity to activate NADP-MDH retained the capacity to activate Fru-P_2ase. Similar stability differences were observed in experiments in which two different thioredoxin fractions were obtained from fresh thioredoxin *c* preparations. However, the stability problems associated with the spinach proteins prevented further progress at this point and prompted us to search for a leaf tissue that would yield more stable preparations.

Light-grown barley seedlings were found to be adequate in this connection. When fractionated by DEAE-cellulose column chromatography, the preparations from barley leaves resembled their spinach counterparts in showing two Fru-P_2ase-linked thioredoxin activities, one that passed through the column (thioredoxin *c*) and one retained on the column that required salt for elution (thioredoxin *f*) (fig.1). Also like spinach, the DEAE-cellulose retentate barley thioredoxin fraction was shown by Sephadex G-75 column chromatography to contain thioredoxin *m* in addition to thioredoxin *f* (fig.2). However, unlike spinach, the thioredoxin *m* was found to be composed of two components, which, in certain preparations, were separated by Sephadex G-75 chromatography. The two thioredoxin *m* components were also separable by electrophoresis in 7.5% polyacrylamide gels (data not shown).

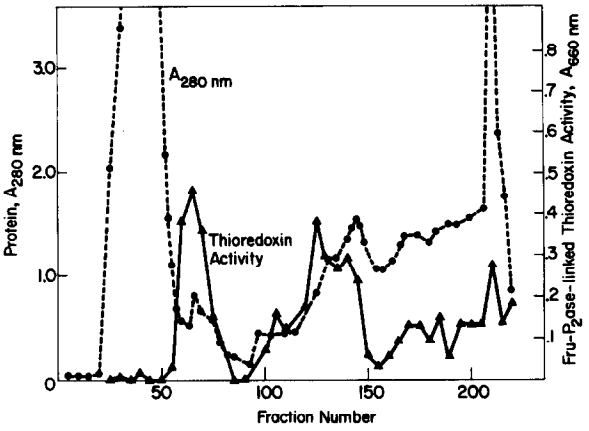

Fig.1. DEAE-cellulose elution profile of Fru-P_2ase-linked thioredoxins from barley leaf extract. The dialyzed acetone precipitate fraction obtained from barley leaves (190 ml) was applied to a 3 × 25 cm DEAE-cellulose column that was equilibrated with buffer A. The column was eluted sequentially with: 350 ml buffer A, 900 ml of a linear gradient (25–200 mM NaCl in buffer A), and 300 ml buffer A containing 500 mM NaCl. Fractions (7.8 ml) were collected at a flow rate of 60 ml/h.

The major new finding with barley concerns the cytoplasmic thioredoxins. As shown in fig.3, the thioredoxin *c* fraction obtained from DEAE-cellulose was resolved by Sephadex G-75 chromatography into

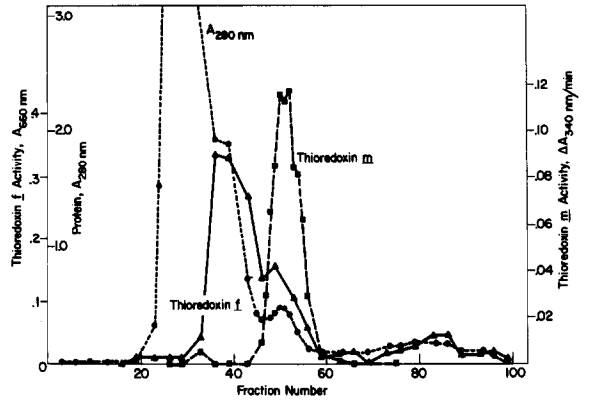

Fig.2. Separation of barley thioredoxins *f* and *m* by Sephadex G-75 column chromatography. The DEAE-cellulose retentate thioredoxin fractions from fig.1 (fractions 115–150) were combined, concentrated to 22 ml by dialysis against solid sucrose and then applied to a 2.5 × 100 cm Sephadex G-75 column equilibrated with buffer A. Fractions of 6 ml were collected at a flow rate of 25 ml/h.

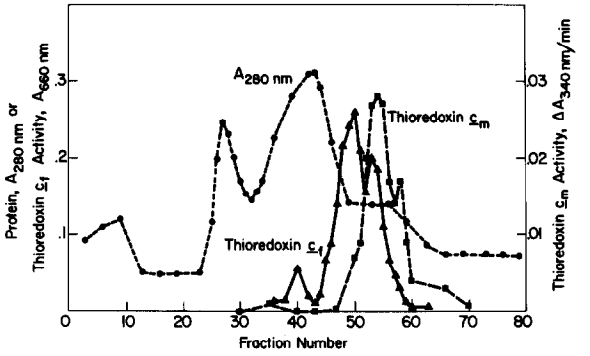

Fig.3. Resolution of barley leaf thioredoxins into c_f and c_m components by Sephadex G-75 column chromatography. The thioredoxin that was eluted from the DEAE-cellulose column just following the void volume in fig.1 (combined fractions 55–80) was concentrated and chromatographed on a Sephadex G-75 column as described in fig.2.

two thioredoxin components which proved to be relatively stable: a component that preferentially activated Fru-P_2ase (thioredoxin c_f) and a component that preferentially activated NADP-MDH (thioredoxin c_m). The relegation of thioredoxins c_f and c_m to the cytoplasm was based on parallel experiments with aqueous extracts of isolated chloroplasts which revealed that in barley, as in spinach, thioredoxins *f* and *m* are located in the chloroplasts, whereas thioredoxins c_f and c_m are not. The finding that the cytoplasmic thioredoxin *c* fraction contains two active components explains our earlier observation that the spinach leaf thioredoxin *c* fraction promoted the light-dependent activation (via ferredoxin and ferredoxin-thioredoxin reductase) of NADP-MDH but not of Fru-P_2ase or other thioredoxin *f*-linked enzymes which were activated by thioredoxin *c* only when reduced chemically by dithiothreitol.

The Sephadex G-75 fractions enriched in each of the chloroplast or cytoplasmic thioredoxins from barley were combined and further purified by hydroxyapatite column chromatography. Table 1 shows that, as was found earlier with thioredoxins *f* and *m* from spinach chloroplasts, the barley cytoplasmic thioredoxins c_f and c_m were separable by hydroxyapatite column chromatography. Hydroxyapatite chromatography was also effective for the later-achieved separation of spinach cytoplasmic thioredoxins c_f and c_m that had been partly separated by Sephadex G-75 chromatography (table 2). From both barley and

Table 1
Fru-P_2ase- and NADP-MDH-linked thioredoxin activity of hydroxyapatite peak fractions of each of the four barley leaf thioredoxins

Thioredoxin	P_i required for elution (mM)	Thioredoxin activity/50 µl		Activity Fru-P_2ase:NADP-MDH
		Fru-P_2ase assay A_{660}	NADP-MDH assay ΔA_{340}/min	
Chloroplast				
Thioredoxin *f*	25	0.13	0.001	130.0
Thioredoxin *m*	5	0.12	0.161	0.7
Cytoplasmic				
Thioredoxin c_f	25	0.13	0.015	9.0
Thioredoxin c_m	5	0.05	0.072	0.7

Fru-P_2ase- and NADP-MDH-linked thioredoxin activity of hydroxyapatite peak fractions of each of the four different barley leaf thioredoxins. Fractions from the Sephadex G-75 chromatography step in fig.2,3 were combined according to their preferential activity (*f* or *m*, c_f or c_m) and were applied individually to a hydroxyapatite column (1.7 × 8 cm for chloroplast thioredoxins; 1.7 × 4 cm for cytoplasmic thioredoxins) that was equilibrated beforehand with buffer A. The column was eluted sequentially with the following concentrations (mM) of potassium phosphate buffer (pH 7.7) in buffer A: 0, 5, 10, 25, 50, 100, 300. Volumes of 100 and 80 ml of each solution were used for the chloroplast and cytoplasmic thioredoxin columns, respectively. The fractions which showed A_{280} were combined for each phosphate concentration. concentrated by dialysis against solid sucrose, and then dialyzed against buffer A for 24 h; the buffer was changed and dialysis was continued for an additional 24 h. Thioredoxin activity was then determined with the Fru-P_2ase or NADP-MDH assays as indicated. Protein concentrations (mg/ml) were: thioredoxin *m*, 2; *f*, 2.3; c_m, 0.4; c_f, 0.3

spinach, thioredoxins *m* and c_m are characterized by a low affinity for hydroxyapatite relative to that of thioredoxins *f* and c_f.

3.2. *Some properties of cytoplasmic thioredoxins* c_f *and* c_m

In addition to differences in enzyme specificity, leaf cytoplasmic thioredoxins c_f and c_m may be distinguished by heat stability and molecular weight. Thioredoxin c_m (and *m*) was more resistant to heat of 80°C than was thioredoxin c_f (and *f*) (table 3). It thus appears that barley thioredoxins of the *m*-type are more heat resistant than are those of the *f*-type. In spinach this is not the case: there, thioredoxins *m* and c_f are heat stable, whereas thioredoxins *f* and c_m are not ([6] and unpublished data).

As for molecular weight, gel exclusion chromatography of the barley cytoplasmic thioredoxins gave mol. wt 20 000 and 15 000 for thioredoxins c_f and c_m, respectively. It is interesting to note that, despite this difference in molecular weight, these two proteins were not separated by electrophoresis in 7.5% polyacrylamide gels. The molecular weights of the chloroplast thioredoxins from barley appeared to be similar to those determined for the corresponding proteins from spinach, i.e., 16 000 for thioredoxin *f* and 9000 for thioredoxin *m*. The second thioredoxin *m* from barley referred to above showed an app. mol. wt 13 000.

4. Concluding remarks

The present results provide evidence that the cytoplasm of leaf cells resembles chloroplasts in containing a thioredoxin of the *m*-type (i.e., specific for NADP-MDH) in addition to a thioredoxin of the *f*-type (i.e., specific for Fru-P_2ase). The finding of an *m*-type thioredoxin in a non-photosynthetic fraction of leaves is of particular interest in view of the demonstration of a seemingly similar type of thioredoxin in preparations from roots [5] and seeds [12]. The function of the non-photosynthetic *m*-type thioredoxins, as well as that of their *f*-type counterparts, remains to be determined.

The physiological mechanism for the reduction of

Table 2
Fru-P_2ase- and NADP-MDH-linked thioredoxin activity of hydroxyapatite peak fractions of each of the four spinach leaf thioredoxins

Thioredoxin	P_i required for elution (mM)	Thioredoxin activity/50 µl		Activity Fru-P_2ase:NADP-MDH
		Fru-P_2ase assay A_{660}	NADP-MDH assay ΔA_{340}/min	
Chloroplast				
Thioredoxin *f*	25	0.42	0.005	84
Thioredoxin *m*	5	0.20	0.061	3
Cytoplasmic				
Thioredoxin c_f	25	0.25	0.001	250
Thioredoxin c_m	5	0.03	0.031	1

Fru-P_2ase- and NADP-MDH-linked thioredoxin activity of hydroxyapatite peak fractions of each of the four different leaf thioredoxins. Fractions from spinach leaves equivalent to those described for barley were subjected to hydroxyapatite column chromatography as given in table 1. Protein concentrations (mg/ml) were: thioredoxin *m*, 0.4; *f*, 0.3; c_m, 1.0; c_f, 1.3

Table 3
Effect of heat on barley leaf thioredoxins

Thioredoxin	% Activity retained in samples heated to 80°C for 4 min
Chloroplast	
Thioredoxin *f*	26
Thioredoxin *m*	113
Cytoplasmic	
Thioredoxin c_f	39
Thioredoxin c_m	100

Effect of heat on barley leaf thioredoxins. Aliquots of the hydroxyapatite fractions enriched in each of the four thioredoxins indicated in table 1 were heated for 4 min at 80°C. Following clarification by centrifugation, the heated samples were assayed for thioredoxin activity with the Fru-P_2ase and NADP-MDH assays

the cytoplasmic thioredoxins is also an unanswered question. This problem assumes renewed importance in view of the recent finding [12] that wheat flour contains an enzyme that catalyzes an NADPH-dependent reduction of thioredoxin like that of the NADP-thioredoxin reductase from animal and bacterial cells. Although chloroplasts (as well as etiolated seedlings [8]) contain a ferredoxin-linked thioredoxin reductase, an NADP-linked thioredoxin reductase from leaves has not been reported. The determination of the reduction mechanism of the leaf cytoplasmic thioredoxins thus joins the elucidation of their functions as an important problem for the future.

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