REGULATION OF NADP-MALATE DEHYDROGENASE BY THE LIGHT-ACTUATED FERREDOXIN/THIOREDOXIN SYSTEM OF CHLOROPLASTS

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1. Introduction

We have recently described a new regulatory system of chloroplasts whereby enzymes are activated in the light by reduction and are deactivated in the dark by oxidation [1]. Activation is achieved in a soluble system that consists of:

(i) Ferredoxin, the strongly electronegative acceptor of photosynthetic electron transport.
(ii) Thioredoxin, a hydrogen carrier protein that is reduced photochemically via ferredoxin.
(iii) Ferredoxin-thioredoxin reductase, a newly-found enzyme that catalyzes the reduction of thioredoxin by photoreduced ferredoxin (eq. 1–3).

$$H_2O + 2\ \text{Ferredoxin}_{oxidized} + \text{Light} \xrightarrow{\text{electron transport chain}} 1/2\ O_2 + 2\ \text{Ferredoxin}_{reduced} \quad (1)$$

$$2\ \text{Ferredoxin}_{reduced} + \text{Thioredoxin}_{oxidized} \xrightarrow{\text{ferredoxin-thioredoxin reductase}} 2\ \text{Ferredoxin}_{oxidized} + \text{Thioredoxin}_{reduced} \quad (2)$$

The thioredoxin reduced photochemically in this manner, in turn, activates selected regulatory enzymes, as indicated in eq. 3.

$$\text{Enzyme}_{inactive} \xrightarrow[\text{thioredoxin}]{\text{reduced}} \text{Enzyme}_{active} \quad (3)$$

The enzymes activated by reduced thioredoxin are deactivated by oxidants indigenous to chloroplasts, such as oxidized glutathione and dehydroascorbate, via a mechanism that may function in vivo in the dark (eq. 4).

$$\text{Enzyme}_{active} \xrightarrow[\text{glutathione}]{\text{oxidized}} \text{Enzyme}_{inactive} \quad (4)$$

Each of the enzymes shown previously to be regulated by the ferredoxin/thioredoxin system is a component of the reductive pentose phosphate cycle for the photosynthetic assimilation of carbon dioxide (fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, phosphoribulokinase, NADP-glyceraldehyde 3-phosphate dehydrogenase) [1,2]. We now report evidence that NADP-malate dehydrogenase (eq. 5), a light-activated enzyme [3–5] not associated with this cycle, is also regulated via the ferredoxin/thioredoxin system.

$$\text{Oxalacetate} + \text{NADPH}_2 \longrightarrow \text{Malate} + \text{NADP} \quad (5)$$

Our results show that both crude and partially purified preparations of NADP-malate dehydrogenase require reduced thioredoxin for activity. The thioredoxin required could be reduced either photochemically with chloroplasts or chemically (in the dark) with the nonphysiological sulfhydryl reagent dithiothreitol. Depending on the mode of activation, NADP-malate dehydrogenase was deactivated either by oxidized glutathione or by an unidentified oxidant

Abbreviation: Fructose 1,6-bisphosphatase, $FruP_2ase$

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formed by chloroplast membranes in the dark. The results are consistent with the view that the ferredoxin/thioredoxin system may be utilized by chloroplasts as a general mechanism for linking light to enzyme regulation.

2. Methods

Chloroplasts were isolated from 100 g greenhouse-grown spinach leaves (that had been harvested and stored in the dark for 1–2 h at 4°C) in a preparative solution containing 350 mM sucrose, 2 mM isoascorbic acid, and 25 mM Hepes buffer (pH 7.6), as described previously [6]. For purification of NADP-malate dehydrogenase, intact chloroplasts were lysed osmotically by suspension in 50 mM Tris–HCl buffer (pH 7.9) (henceforth called 'buffer') to a chlorophyll concentration of 1 mg/ml [7]. Chloroplast membrane fragments were removed by centrifugation (15 min, 40 000 × *g*) and the supernatant fraction ('chloroplast extract') [8] was applied to a DEAE-cellulose column (0.5 × 10 cm) that had been equilibrated beforehand with buffer. The protein fraction that passed through the column was collected. With certain lots of leaves, the enzyme remained on the column and was eluted with buffer containing 100 mM NaCl. Such preparations were also largely free of thioredoxin.

Solid ammonium sulfate was added to the column effluent to 40% saturation, the greenish precipitate was removed by centrifugation as above, and ammonium sulfate was added to the supernatant fraction to 55% saturation. The suspension was centrifuged as before, the supernatant fraction was discarded, and the pellet was dissolved in 1–2 ml buffer. This solution, which contained thioredoxin-free NADP-malate dehydrogenase, was dialyzed for 2 h against 1 liter of buffer. The dialyzed preparation was used as the source of NADP-malate dehydrogenase in the experiments described below unless indicated otherwise. The enzyme could be stored at −20°C for several days with little loss of activity. Chloroplast thioredoxin and ferredoxin-thioredoxin reductase were prepared as described previously [1]. *Escherichia coli* thioredoxin was partially purified by heating for 3 min at 80°C a pH 4.5 supernatant fraction obtained as indicated previously [9].

3. Results and discussion

3.1. *Activation of NADP-malate dehydrogenase by dithiothreitol-reduced thioredoxin*

In agreement with the findings of others [3,4,10,11], we observed that both crude and partially purified preparations of chloroplast NADP-malate dehydrogenase are inactive in the absence of a low-potential reductant, such as dithiothreitol. The function of the thiol became apparent in our studies once thioredoxin was added to the assay mixture. As shown in fig.1, chloroplast thioredoxin added to either chloroplast extract or a partially purified enzyme preparation in the presence of dithiothreitol markedly increased NADP-malate dehydrogenase activity. By analogy with other enzymes, it would appear that the dithiothreitol is needed to reduce thioredoxin which, in turn, activates NADP-malate dehydrogenase. It is noteworthy that chloroplast NAD-linked malate dehydrogenase, unlike the NADP-linked counterpart, was unaffected by thioredoxin under these conditions.

Studies with the partially purified NADP-malate dehydrogenase (from which endogenous thioredoxin was removed) revealed that chloroplast thioredoxin could be replaced by its counterpart from *E. coli*. As is the case for other thioredoxin-dependent enzymes of chloroplasts, sulfhydryl reagents such as reduced glutathione and 2-mercaptoethanol could not replace dithiothreitol with either the unfractionated or the purified NADP-malate dehydrogenase preparation.

As was found previously for the regulatory enzymes of CO_2 assimilation [1,12–14], the rate of activation of NADP-malate dehydrogenase was slow relative to the rate of catalysis. Accordingly, to obtain maximal activity, the enzyme was preincubated with reduced thioredoxin prior to measuring catalytic activity. Figure 2 shows the effect of time of preincubation on the activity of NADP-malate dehydrogenase when activation and catalysis were both measured under optimal conditions. Activity of the enzyme rose progressively as a function of preincubation time throughout the 10 min interval tested and, as shown in separate experiments, was unaffected by the presence of $MgSO_4$ at concentrations ranging from 1–10 mM (cf. refs [3,4]). It is significant that, even with prolonged preincubation periods, the activation of NADP-malate dehydrogenase by dithiothreitol was

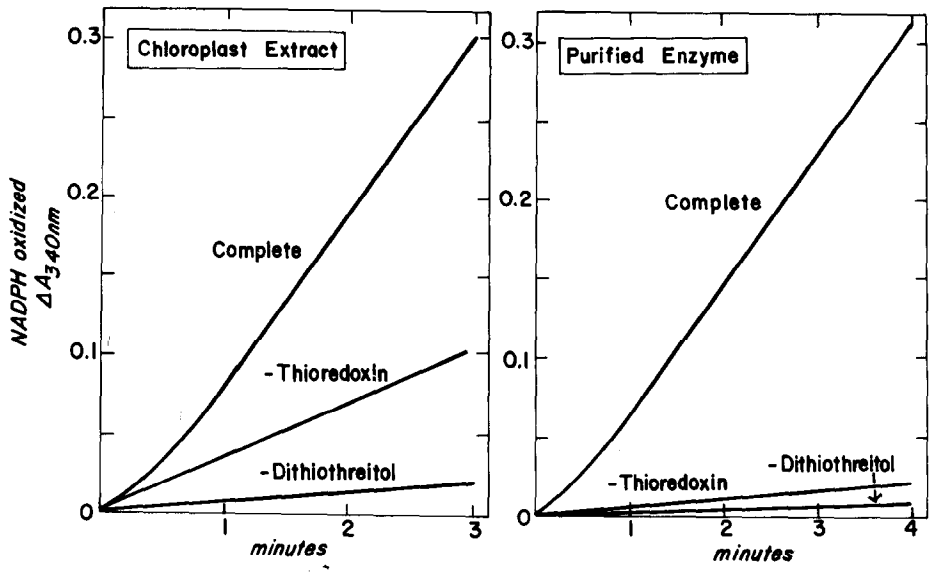

Fig.1. Activation of chloroplast NADP-malate dehydrogenase by dithiothreitol-reduced thioredoxin. The complete system (in a cuvette of 1 cm light path) contained 25 μg chloroplast thioredoxin, chloroplast extract equivalent to 0.2 mg chlorophyll or 0.12 mg partially purified malate dehydrogenase, and the following: Tris–HCl buffer (pH 7.9), 100 μmol; dithiothreitol, 5 μmol NADPH, 0.25 μmol. Vol. 0.9 ml. After preincubation for 5 min, 0.1 ml (2.5 μmol) oxalacetic acid was added and the change in absorbance at 340 nm was followed with a Cary 14 spectrophotometer. Temperature, 22°C.

strictly dependent on thioredoxin. For example, a control (minus thioredoxin) sample preincubated with dithiothreitol alone for 10 min showed only 5% of the activity displayed by the complete (plus thioredoxin) sample shown in fig.2. The response of the purified NADP-malate dehydrogenase to different concentrations of added thioredoxin during a 5 min preincubation period is shown in fig.3. These results indicate that the thioredoxin requirement for NADP-malate dehydrogenase is higher than that of other chloroplast enzymes that have been investigated. A further point suggested by these results is that the protein factor that was isolated by Jacquot et al. [11] and shown to be required for dithiothreitol-dependent activation of NADP-malate dehydrogenase is identical with thioredoxin.

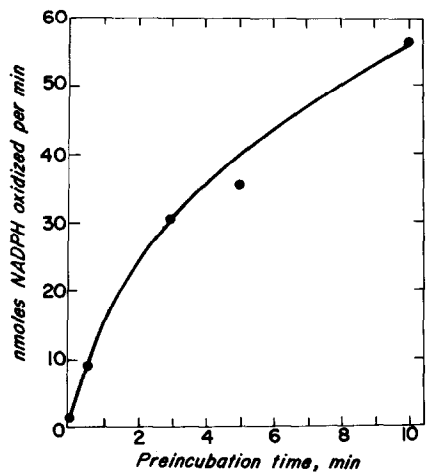

Fig.2. Effect of time of preincubation on the activation of chloroplast NADP-malate dehydrogenase by dithiothreitol-reduced thioredoxin. Partially purified enzyme (320 μg) was preincubated in 0.1 ml of a solution containing 25 μg chloroplast thioredoxin, 10 μmol Tris–HCl buffer (pH 7.9), and 1 μmol dithiothreitol. After preincubation, the mixture was injected into a 1 cm cuvette of 1 ml capacity that contained: Tris–HCl buffer (pH 7.9), 100 μmol and NADPH, 0.25 μmol. The reaction was started by the addition of 2.5 μmol of oxalacetic acid. The change in absorbance at 340 nm was followed with a Cary 14 spectrophotometer. Temperature, 22°C.

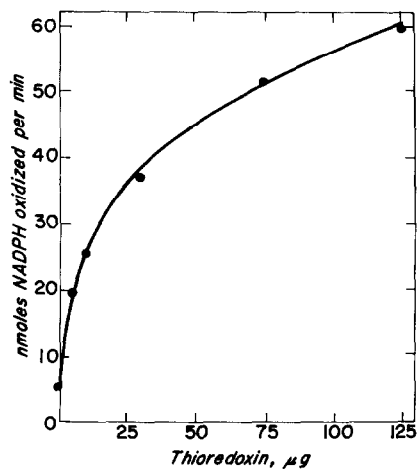

Fig.3. Effect of thioredoxin concentration on chloroplast NADP-malate dehydrogenase in the presence of dithiothreitol. Except for varying the thioredoxin concentration, experimental conditions were as described in fig.2.

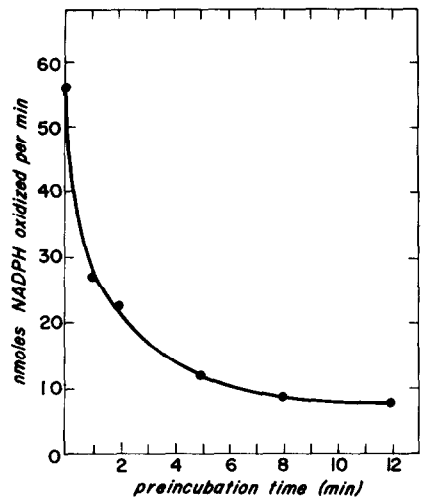

Fig.4. Effect of time of preincubation with oxidized glutathione on the deactivation of chloroplast NADP-malate dehydrogenase activated by dithiothreitol-reduced thioredoxin. Chloroplast NADP-malate dehydrogenase was activated for 5 min under the conditions in fig.2, 1.5 μmol oxidized glutathione was added, and preincubation was continued for the indicated times. NADP-malate dehydrogenase activity was then measured as described in fig.2.

3.2. *Deactivation of NADP-malate dehydrogenase activated by dithiothreitol-reduced thioredoxin*

One of the distinctive features of chloroplast enzymes that are activated by reduced thioredoxin is their capacity for deactivation by an added oxidant [1]. With dithiothreitol as reductant, NADP-malate dehydrogenase was no exception to this rule. The enzyme activated by thioredoxin in the presence of dithiothreitol was deactivated on incubation with oxidized glutathione (fig.4). Deactivation of NADP-malate dehydrogenase was a function of the time that the enzyme was exposed to the oxidant, i.e., the longer the time of preincubation, the more complete the deactivation. After a 1 min preincubation of the activated enzyme with oxidized glutathione, activity was about 50% that of the control – a value that decreased to 5% ater a 5 min preincubation.

3.3. *Regulation of NADP-malate dehydrogenase by the ferredoxin/thioredoxin system*

The finding that thioredoxin, reduced chemically in the dark with dithiothreitol, activates chloroplast NADP-malate dehydrogenase raises the question whether photochemically reduced thioredoxin can achieve the same effect. The results of table 1 speak to this question in the affirmative. When tested in the chloroplast system for reducing thioredoxin [1], NADP-malate dehydrogenase was activated in a reaction that was strictly dependent on light, on thioredoxin, and on the components required for the photochemical reduction on thioredoxin (ferredoxin and ferredoxin-thioredoxin reductase). There was no evidence for a light-induced activation of the enzyme by chloroplast membranes in the absence of ferredoxin (cf. ref. [5]).

Chloroplast NADP-malate dehydrogenase thus shows a striking resemblance to $FruP_2$ase [1,2,15] and to other regulatory enzymes of CO_2 assimilation in chloroplasts in its capacity for activation via the ferredoxin/thioredoxin system. By contrast, the mechanism for the 'dark deactivation' of photochemically activated NADP-malate dehydrogenase is basically different from that for $FruP_2$ase and other enzymes that have been investigated. Unlike the deactivation described in fig.4 for dithiothreitol/thioredoxin, the decativation of NADP-malate dehydrogenase that had been activated in the light via the ferredoxin/thioredoxin system occurred in the dark rapidly, as reported for the enzyme

Table 1
Requirements for the photochemical activation of chloroplast NADP-malate

Treatment	$NADPH_2$ oxidized (nmol/min)
Light, complete	15
Light, minus thioredoxin	3
Light, minus ferredoxin	1
Light, minus ferredoxin-thioredoxin reductase	2
Light, minus NADP-malate dehydrogenase	2
Dark, complete	1

The reaction was executed in Warburg-Krippahl vessels containing (in the sidearm) 3.6 mg partially purified chloroplast NADP-malate dehydrogenase and (in the central compartment) 60 μg spinach ferredoxin, 125 μg chloroplast thioredoxin, 12 μg ferredoxin-thioredoxin reductase, twice-washed spinach chloroplast fragments equivalent to 10 μg chlorophyll and 20 μmol Tris–HCl buffer (pH 7.9). Temperature, 20°C. Vessels were equilibratde for 6 min with nitrogen. NADP-malate dehydrogenase was added from the sidearm, and the enzyme was activated by a 10 min illumination (20 000 lux). Final vol. 0.4 ml. After preincubation, 0.2 ml of the mixture containing the activated NADP-malate dehydrogenase was injected into the assay solution that contained in vol. 0.8 ml, 100 μmol Tris–HCl buffer (pH 7.9) and 0.25 μmol NADPH. Following the addition of 2.5 μmol oxalacetic acid, the change in absorbance at 340 nm was followed as in fig.2

in situ [3,5]. Furthermore, such deactivation induced by darkness was independent of an added oxidant and was reversible, i.e., the deactivated enzyme was again activated when light was restored (table 2). The identity of the oxidant that functions in the deactivation of malate dehydrogenase under dark conditions is not known, but, as shown in table 3, the oxidant appears to be bound to the chloroplast membranes and to be available for deactivation only when the membranes are maintained in the dark.

Table 2
Deactivation in the dark of chloroplast NADP-malate dehydrogenase activated in the light by the ferredoxin/thioredoxin system

Treatment	$NADPH_2$ oxidized (nmol/min)
15 min, dark	2
15 min, light	68
15 min, light, 1 min, dark	14
15 min, light, 1 min, dark, 5 min, light	58

Except for the time and the indicated differences during preillumination (activation), experimental conditions were as in table 1

Table 3
Evidence for a chloroplast membrane-bound component functional in the dark deactivation of NADP-malate dehydrogenase activated by dithiothreitol-reduced thioredoxin

Deactivation factor added	$NADPH_2$ oxidized (nmol/min)
None	34
Oxidized glutathione	16
Chloroplast membranes (dark)	16
Chloroplast membranes (light)	33

A partially purified NADP-malate dehydrogenase preparation was activated by a 10 min preincubation with dithiothreitol and thioredoxin as described in fig.2. Oxidized glutathione (5 μmol) or twice-washed chloroplast membranes (200 μg chlorophyll) were then added as indicated. The preincubation was continued for 2 min, and NADP-malate dehydrogenase was assayed as described in fig.2. For the dark treatment, membranes were kept in the dark at 0°C for 3 min and were added to the preincubation mixture that was maintained in the dark for the 2 min indicated above. For the light treatment, these same 'dark' membranes were illuminated for 2 min prior to addition to the preincubation mixture, which was kept in the light for 2 min prior to addition to the assay mixture

4. Concluding remarks

The current findings constitute evidence that reduced thioredoxin functions in the regulation of NADP-malate dehydrogenase – a chloroplast enzyme that is not associated with the reductive pentose phosphate cycle of photosynthetic carbon dioxide assimilation. Like other thioredoxin-linked regulatory enzymes of chloroplasts, the NADP-malate dehydrogenase reaction consists of two phases which differ in rate:

(i) An activation phase in which reduced thioredoxin changes the enzyme from an inactive to an active form.

(ii) A catalytic phase in which the activated enzyme converts reactants to products.

As is the case for enzymes of the carbon reduction cycle, activation is slow relative to the rate of catalysis. However, by contrast, the activation and catalytic phases of NADP-malate dehydrogenase are independent of transient changes in the concentration of Mg^{2+} and of metabolites which could serve as enzyme effectors.

The thioredoxin required by NADP-malate dehydrogenase can be reduced either chemically with the non-physiological sulfhydryl reagent dithiothreitol or photochemically with the physiological ferredoxin and ferredoxin-thioredoxin reductase system. The activated enzyme appears to be deactivated rapidly by a chloroplast membrane-bound component that is oxidized only in the dark. The results thus suggest that the in vivo activity of NADP-malate dehydrogenase is controlled by a balance between the light-actuated ferredoxin/thioredoxin system for activation and the dark-dependent (as yet unidentified) oxidant required for deactivation.

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References

[1] Wolosiuk, R. A. and Buchanan, B. B. (1977) Nature 266, 565–567.
[2] Schürmann, P., Wolosiuk, R. A., Breazeale, V. D. and Buchanan, B. B. (1976) Nature 263, 257–258.
[3] Johnson, H. S. and Hatch, M. D. (1970) Biochem. J. 119, 273–280.
[4] Johnson, H. S. (1971) Biochem. Biophys. Res. Commun. 43, 703–709.
[5] Anderson, L. E. and Avron, M. (1976) Plant Physiol. 57, 209–213.
[6] Kalberer, P. P., Buchanan, B. B. and Arnon, D. I. (1967) Proc. Natl. Acad. Sci. USA 67, 1542–1549.
[7] Arnon, D. I. (1949) Plant Physiol. 24, 1–15.
[8] Buchanan, B. B., Kalberer, P. P. and Arnon, D. I. (1967) Biochem. Biophys. Res. Commun. 29, 74–79.
[9] Buchanan, B. B. and Wolosiuk, R. A. (1976) Nature 264, 669–670.
[10] Ting, I. P. and Rocha, V. (1971) Arch. Biochem. Biophys. 147, 156–164.
[11] Jacquot, J. P., Vidal, J. and Gadal, P. (1976) FEBS Lett. 71, 223–227.
[12] Wolosiuk, R. A. and Buchanan, B. B. (1976) J. Biol. Chem. 251, 6456–6461.
[13] Schürmann, P. and Wolosiuk, R. A. (1977) submitted.
[14] Lorimer, G. H., Badger, M. R. and Andrews, T. J. (1976) Biochemistry 15, 529–536.
[15] Buchanan, B. B., Schürmann, P. and Kalberer, P. (1971) J. Biol. Chem. 246, 5952–5959.