Biochimica et Biophysica Acta, 636 (1981) 58–64
Elsevier/North-Holland Biomedical Press

BBA 48063

DARK MODULATION OF NADP-DEPENDENT MALATE DEHYDROGENASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN THE CHLOROPLAST

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(Received August 1st, 1980)
(Revised manuscript received January 19th, 1981)

Key words: Dark modulation; Thioredoxin; Malate dehydrogenase; Glucose-6-phosphate dehydrogenase; (Pea chloroplast)

The properties of the system which reverses light modulation of NADP-dependent malate dehydrogenase and glucose-6-phosphate dehydrogenase activity in pea chloroplasts were examined. A factor catalyzing dark modulation of these enzymes was found. This factor cochromatographed with thioredoxin in all systems used (Sephacryl S-200, Sephadex G-75, DEAE-cellulose). Inhibition of dithiothreitol-dependent modulation and of dark reversal by antibody against *Escherichia coli* thioredoxin further suggest that the dark factor is in fact thioredoxin. It appears that the reaction is the reverse of the previously described dithiothreitol-dependent thioredoxin-catalyzed modulation of enzymes. The limiting step in vitro seems to be the oxidation of thioredoxin during the dark period.

Introduction

Upon illumination the activity of several enzymes located in the chloroplast stroma is changed (see Ref. 1). Electrons can be provided either by the photosynthetic electron-transport chain located in the thylakoid membrane or by a strong reducing agent such as dithiothreitol; both reactions cause modulation of enzyme activity. Although a slow in vitro reduction by dithiothreitol can be demonstrated using partially purified NADP-dependent malate dehydrogenase (EC 1.1.1.82) or glucose-6-phosphate dehydrogenase (EC 1.1.1.49), the rate is increased markedly upon addition of thioredoxin. This soluble protein has been demonstrated to mediate the ferredoxin-dependent activation via ferredoxin-thioredoxin reductase using irradiated thylakoid membranes as the electron source [2]. Ashton and Anderson [3] and Lara et al. [4,5] have recently reported the presence of another stromal protein factor which can mediate light modulation in the absence of ferredoxin, the reductase and thioredoxin. Although evidence for several different mechanisms [6–9] for light modulation has been obtained, it has not yet been conclusively demonstrated which is significant physiologically.

In order to provide a useful means for the regulation of CO_2 fixation and starch breakdown in the chloroplast, the light-modulation reaction(s) must be reversible upon exposure to darkness. That this happens has already been shown earlier for leaf extracts [10] and for isolated intact chloroplasts [6,11] as well as for a broken chloroplast system [12].

Little is known about the factors influencing dark modulation of these enzymes. Several small non-protein compounds such as oxidized glutathione and dehydroascorbate, which are known to be present in the chloroplast, have been suggested to be responsible for dark modulation of fructose-1,6-bisphosphatase (EC 3.1.3.11) [13]. However, Halliwell and Foyer [14] have shown that glutathione is most

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unlikely to be the physiological oxidant. On the other hand, an unidentified membrane-bound oxidant has been assumed to be the deactivator of NADP-dependent malate dehydrogenase [2]. H_2O_2 might also be the oxidant for SH-group-containing enzymes [15]. In fact, Brennan and Anderson [16] showed an inhibitory effect of catalase on the dark modulation of light-inactivated glucose-6-phosphate dehydrogenase in a broken chloroplast system. But since H_2O_2 is generated during illumination rather than in the dark in broken chloroplast systems it seems unlikely that there is prolonged oxidation by H_2O_2 in the dark. The in vitro inactivation of dithiothreitol-activated NADP-dependent malate dehydrogenase seems to be oxygen dependent and is enhanced by a heat-stable protein factor ('regulatory protein') [17] which might be identical with thioredoxin. Recently, it has been demonstrated for fructose-1, 6-bisphosphatase that the availability of electrons determines whether the enzyme is active [18]. This finding corroborates strongly our view of the modulation system.

Materials and Methods

Pea (*Pisum sativum* L., var. Little Marvel) plants were grown in vermiculite in a greenhouse for 10–14 days.

Chloroplasts were prepared according to the method of Cockburn et al. [19], except that sodium isoascorbate was omitted. Intact chloroplasts were resuspended and washed once in an ascorbate-free isotonic medium according to the method of Stokes and Walker [20]. Broken chloroplasts were obtained by resuspension of once-washed intact chloroplasts in 50 mM Hepes-KOH, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl (hypotonic buffer) using a Ten Broeck glass homogenizer. The stromal fraction was obtained by centrifugation of the homogenized chloroplast suspension at 15 000 × *g* for 15 min. The resuspended and homogenized pellet was used as the unwashed particulate fraction. Washing steps (one, if not indicated separately) were performed using an excess of hypotonic buffer followed by homogenization and centrifugation at 15 000 × *g*.

The buffer used for all enzyme assays was 100 mM Tris-HCl, pH 8.0, 1 mM EDTA. The other components present in the reaction mixture were as follows: glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 1 mM glucose-6-phosphate, 0.25 mM $NADP^+$, NADP-dependent malate dehydrogenase (EC 1.1.1.82), 10 mM oxaloacetate, 0.2 mM NADPH. The enzyme activities were followed in the 1 ml reaction mixture at room temperature at 340 nm using either a Cary 219 or a Gilford 2400 recording spectrophotometer. Thioredoxin activity was determined by incubation of partially purified NADP-dependent malate dehydrogenase (or glucose-6-phosphate dehydrogenase) with 10 mM dithiothreitol [21] and the initial rate of activation (or inactivation) was used as a means to calculate relative activity.

Chlorophyll content was determined from A_{665} and A_{649} readings in 80% acetone [22]. Protein concentration was estimated according to the method of Lowry et al. [23].

For the partial purification of glucose-6-phosphate dehydrogenase and NADP-dependent malate dehydrogenase from the chloroplast stromal fraction, the procedure described by Ashton and Anderson [3] has been used. As a source for partially purified thioredoxin, those fractions obtained after gel filtration of the 40–75% saturated $(NH_4)_2SO_4$ precipitate which showed the ability to enhance the dithiothreitol-dependent activation of NADP-dependent malate dehydrogenase or inactivation of glucose-6-phosphate dehydrogenase were used. These fractions were placed in a dialysis bag and covered by solid sorbitol to concentrate the sample, then dialyzed and chromatographed on DEAE-cellulose (column dimensions, 2.65 cm^2 × 10 cm) using 25 mM Hepes-KOH, pH 7.0, 5 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA for washing and a linear gradient from 0 to 0.2 M KCl in the same buffer for elution. After heat treatment (10 min, 80°C) of the diluted protein solution, gel filtration over Sephadex G-75 (2.0 cm^2 × 42 cm) and another DEAE-cellulose (2.65 cm^2 × 10 cm) step were performed.

In order to obtain light-modulated enzyme, partially purified NADP-dependent malate dehydrogenase or glucose-6-phosphate dehydrogenase, protein modulase [3], and washed thylakoid membranes (0.1–0.2 mg chlorophyll/ml) were illuminated ($59 \cdot 10^3$ lx) for 15 min in a waterbath at 25°C.

After 10-fold dilution with ice-cold deionized water the particulate fraction was removed by centrifugation (15 000 × *g*, 10 min). The glucose-6-

phosphate dehydrogenase in the supernatant fluid which was kept on ice exhibited only a slow change in activity due to spontaneous reactivation and could be used for several hours. The NADP-dependent malate dehydrogenase activity, however, decayed more rapidly. The actual activity was determined each time immediately before it was used for a dark-modulation experiment.

In vitro activation (inactivation) by dithiothreitol (100 mM) of partially purified NADP-dependent malate dehydrogenase (glucose-6-phosphate dehydrogenase) was performed by incubation for 1 h at room temperature. Before using the modulated enzyme in dark-modulation experiments, the dithiothreitol was removed by gel filtration through Sephadex G-25 (0.38 $cm^2 \times 17$ cm) with N_2-flushed buffer. The modulated enzyme was well separated from the dithiothreitol peak. Dithiothreitol was determined by means of its reaction with 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 50 mM Tris-HCl, pH 8.9. The enzyme was kept under N_2 until use. The antibody preparation against *Escherichia coli* thioredoxin [24] was a generous gift from A. Holmgren.

Biochemicals were products of Sigma Chemical Co. Other chemicals were analytical grade reagents or the highest grade commercially available. Pea seeds were obtained from Northrup and King, Chicago.

Results and Discussion

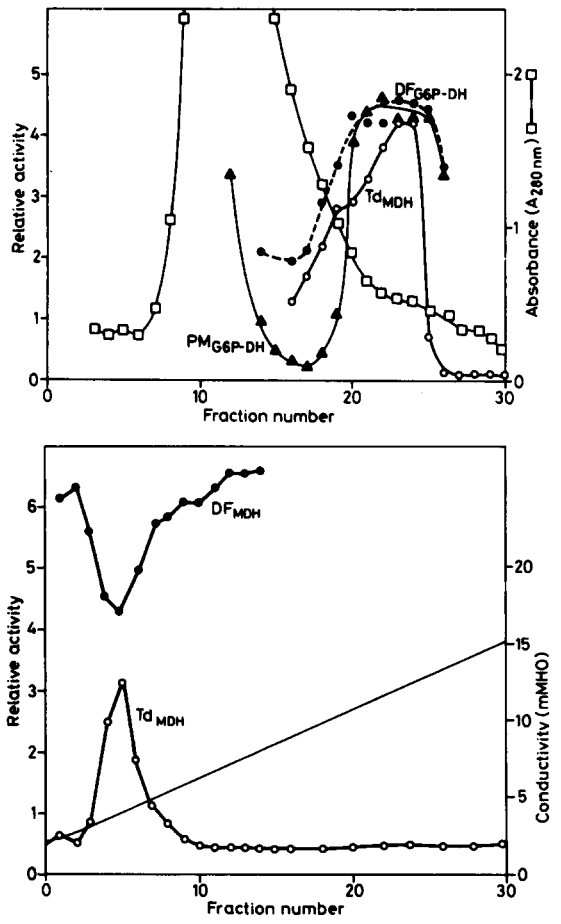
Passage of the 40–75% saturated $(NH_4)_2SO_4$ fraction prepared from the chloroplast stroma through Sephacryl S-200 (1.1 $cm^2 \times 54$ cm) resulted in the separation of NADP-dependent malate dehydrogenase, protein modulase, which catalyzes the light modulation of several enzymes in the presence of thylakoid membranes, and thioredoxin [3]. The latter eluted in a broad peak. No attempt was made to separate the different thioredoxin forms as reported by Wolosiuk et al. [25]. Thioredoxin activity was determined as described by Ashton et al. [21] using its ability to catalyze the activation of NADP-dependent malate dehydrogenase or the inactivation of glucose-6-phosphate dehydrogenase by dithiothreitol. The Sephacryl S-200 profile (Fig. 1) was scanned for fractions enhancing dark modulation, i.e., for fractions that could catalyze the activation of light-inactivated glucose-6-phosphate dehydrogenase and the inactivation of light- or dithiothreitol-activated NADP-dependent malate dehydrogenase (not shown in Fig. 1). A peak of such activity comigrated with thioredoxin and will be called dark factor. No thylakoid membranes are required for the function of the partially purified soluble dark factor.

Further purification steps were applied in order to establish that the thioredoxin fractions also retained the ability to enhance dark modulation. Coelution of both activities from DEAE-cellulose is shown in Fig. 2. Subsequent filtration of the fractions through Sephadex G-75 failed to resolve both activities (Fig. 3). After further purification by heat treatment (10 min, 80°C) and rechromatography on Sephadex G-75 both activities eluted in an identical position (data not shown). Using the methods listed above the two activities apparently cannot be separated. Since no reducing agent was added during the purification procedure, thioredoxin was probably obtained in its oxidized form. According to these results it can be assumed that thioredoxin catalyzes the dark modulation of NADP-dependent malate dehydrogenase and glucose-6-phosphate dehydrogenase.

The dark modulation of NADP-dependent malate dehydrogenase [6,11] and glucose-6-phosphate dehydrogenase [6] has been shown to be very rapid in intact chloroplasts. In the broken or recombined chloroplast system the reaction could be delayed by 5–10-fold dilution with hypotonic buffer. Thus, it was possible to follow the time course of inactivation experimentally.

Partially purified thioredoxin-free NADP-dependent malate dehydrogenase, activated by dithiothreitol and treated as described in Materials and Methods to remove dithiothreitol, can be inactivated by addition of oxidized (see above) thioredoxin. The initial rate of inactivation is very rapid. The amount of added thioredoxin determines the extent of inactivation. Also, in a system consisting of stroma and washed thylakoid membranes, the extent of inactivation is directly proportional to the amount of added thioredoxin when limiting amounts of this compound are used (Fig. 4).

NADP-dependent malate dehydrogenase, activated



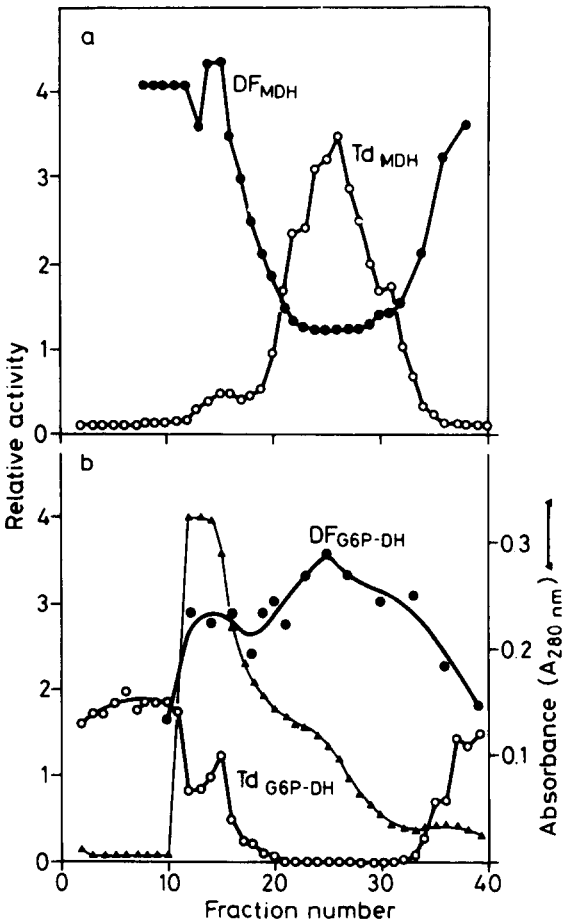


Fig. 1 (Top left). Sephacryl S-200 gel filtration profile of a stromal fraction (40–75% saturated $(NH_4)_2SO_4$). The activity was determined as follows. Protein modulase (PM) (▲———▲): 100 µl of each fraction were incubated with 20 µl of twice-washed thylakoids membranes (corresponding to 28 µg of chlorophyll) and 50 µl of partially purified glucose-6-phosphate dehydrogenase (G6P-DH) in the light ($59 \cdot 10^3$ lx). After 10 min an aliquot was diluted 5-fold with 10 mM Tris-HCl, pH 9.0. Thylakoid membranes were removed immediately by centrifugation (1 min) in a microcentrifuge. The activity was determined in the supernatant fluid. Thioredoxin (Td) (○———○): 50 µl of each fraction were incubated with 20 µl of partially purified NADP-dependent malate dehydrogenase and 10 µl of 0.5 M dithiothreitol. Activity was determined after 5 min. Dark factor (DF) (●———●): Incubation conditions as for protein modulase. Activity after 10 min dark treatment is used as a measure for dark factor activity. Activities are given in arbitrary units. □———□, protein (A_{280}). MDH, malate dehydrogenase.

Fig. 2. (Bottom left). DEAE-cellulose chromatography. The pooled Td/DF fractions from the Sephacryl S-200 (Fig. 1) profile were dialysed and concentrated (Material and Methods) by removal of water with sorbitol external to the dialysis bag. A linear gradient from 0 to 0.25 M KCl in 25 mM Hepes-KOH, pH 6.3, 5 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA was used for elution. Fractions of 70 drops (4.9 ml) were collected. The activities were determined as follows: Thioredoxin (Td) (○———○) was detected as described in Fig. 1. Dark factor (DF) (●———●): 50 µl of each fraction were incubated with partially purified, dithiothreitol-treated NADP-dependent malate dehydrogenase (dithiothreitol removed) for 3 min. Activity was determined immediately. MDH, malate dehydrogenase.

Fig. 3 (Right). Sephadex G-75 gel filtration. The preceding purification steps were $(NH_4)_2SO_4$ fractionation (40–75% saturated) and DEAE-cellulose chromatography of a stromal extract. Fractions with thioredoxin and dark factor activity were pooled after the DEAE-cellulose (3.9 cm^2 × 18 cm) step, dialysed and concentrated by removal of water with solid sorbitol external to the dialysis bag. Fractions of 28 drops (1.96 ml) were collected. The activities were determined as described in the legend of Fig. 1 using the corresponding partially purified enzyme: (a) NADP-dependent malate dehydrogenase (MDH); (b) glucose-6-phosphate dehydrogenase (G6P-DH); ▲———▲, protein (A_{280}). DF, dark factor, Td, thioredoxin.

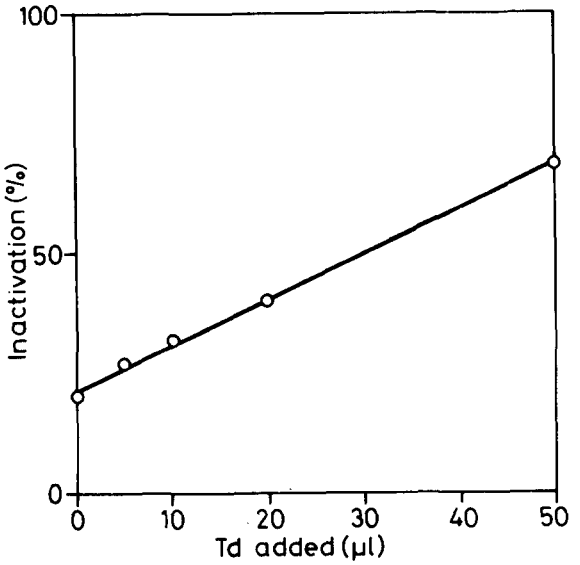


Fig. 4. Inactivation of light-activated NADP-dependent malate dehydrogenase upon addition of exogenous thioredoxin (Td) in a recombined chloroplast system. Light modulation was achieved by keeping 2 parts of stroma and 1 part of washed thylakoid membranes under continuous light ($59 \cdot 10^3$ lx) (corresponding to 113 µg of chlorophyll/ml). Maximal activity was 85 µmol oxaloacetate reduced/mg chlorophyll per h (using the amount of chlorophyll equivalent to the stroma present in the incubation mixture as the basis for calculation of enzyme activity). Aliquots were withdrawn, diluted 10-fold with buffer (containing the indicated amounts of thioredoxin) and transferred to the dark. Samples were taken and the change of activity during the first minute was used to calculate the inactivating effect of thioredoxin. 10 µl of thioredoxin correspond to 53 µg of protein.

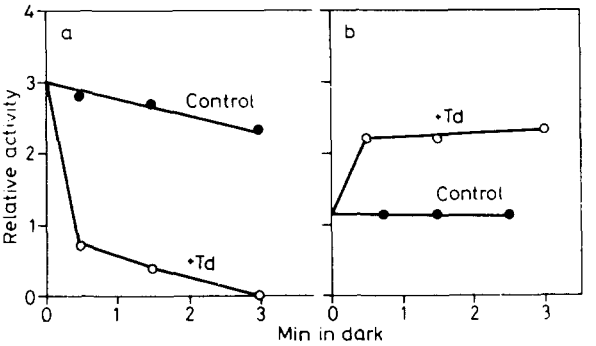


Fig. 5. Dark modulation of partially purified, light-modulated NADP-dependent malate dehydrogenase (a) and glucose-6-phosphate dehydrogenase (b) by oxidized thioredoxin (Td). Light modulation was achieved by incubating partially purified enzyme, protein modulase and washed thylakoid membranes for 15 min. The reaction was stopped by 10-fold dilution with cold deionized water. Thylakoid membranes were removed by centrifugation. The supernatant fluid served as the source of light-modulated enzyme. For dark modulation 2 parts of enzyme solution were combined with 1 part of oxidized thioredoxin solution or buffer (control) and aliquots were assayed for activity. The presence of washed membranes caused no additional effect on dark modulation.

by light in the presence of protein modulase and washed thylakoid membranes as described in Materials and Methods, can also be inactivated by thioredoxin-containing fractions (Fig. 5a). Similarly, partially purified, light-inactivated glucose-6-phosphate dehydrogenase was rapidly and completely reactivated upon addition of thioredoxin (Fig. 5b).

As can be predicted from the data given in Fig. 4, the addition of exogenous oxidized thioredoxin immediately before the dark period causes a rapid decrease of NADP-dependent malate dehydrogenase activity in a light-treated recombined chloroplast system (Fig. 6). The presence of the additional thioredoxin during the light period, however, leads to a stimulated light activation rate of the enzyme (not shown). Therefore, it has to be assumed that thioredoxin becomes reduced during illumination. The additional thioredoxin also leads to an enhanced dark inactivation rate as compared to the rate obtained without exogenous thioredoxin. Comparing this enhanced rate, however, with the rapid one obtained upon addition of an equal amount of oxidized thioredoxin after the light period (no reduction) shows that the oxidation of the reduced thioredoxin in the dark must be the rate-limiting step in the diluted in vitro system.

Although the major part of chloroplast thioredoxin is found as a soluble stromal protein, about 5% of the total thioredoxin has been reported to be bound tightly to the thylakoid membranes [21]. Using the reported method, this thioredoxin activity has been extracted with 1% Triton X-100 and partially purified. This solubilized thioredoxin exhibits dark factor activity comparable to that of the soluble form (data not shown).

Further experiments were performed to confirm the identity of the dark factor as thioredoxin. An

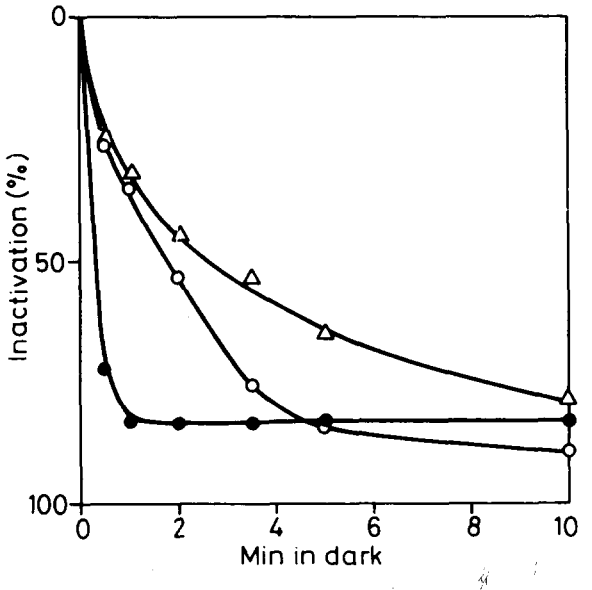


Fig. 6. Dark inactivation of NADP-dependent malate dehydrogenase in a recombined chloroplast system in the presence of oxidized and reduced thioredoxin. △———△, no exogenous thioredoxin added; ○———○, exogenous thioredoxin was present during the preceding light period and had been reduced; ●———●, exogenous thioredoxin (oxidized) was added only for the dark period. For light activation 1 part of stroma and 1 part of washed membranes were incubated for 20 min (chlorophyll concentration, 280 μg/ml). NADP-dependent malate dehydrogenase activity in the absence of thioredoxin was 31 μmol oxaloacetate reduced/mg chlorophyll per h, in the presence of thioredoxin 80 μmol oxaloacetate/mg chlorophyll per h. Dark treatment was started immediately by diluting an aliquot volume 5-fold with buffer. Samples were taken at the indicated times and activity was determined.

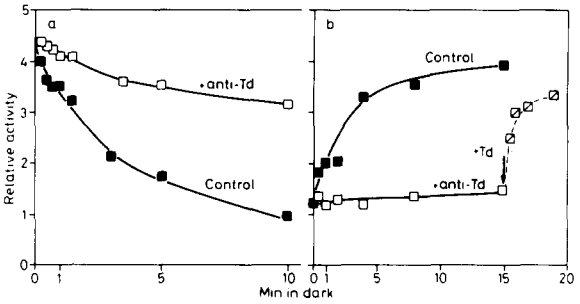


Fig. 7. Inhibition of dark modulation of light-treated NADP-dependent malate dehydrogenase (a) and glucose-6-phosphate dehydrogenase (b) in a recombined chloroplast system. (a) For light treatment and activity in the light see legend to Fig. 4. Dilution in the dark was 5-fold in hypotonic buffer containing thioredoxin-antibody (anti-Td) (▫———▫) or preimmune serume (control) (▪———▪), respectively. (b) 10 parts of stroma (enriched with partially purified glucose-6-phosphate dehydrogenase and 1 part of washed thylakoid membranes incubated in the light ($59 \cdot 10^3$ lx) for 10 min (chlorophyll concentration, 35.6 μg/ml). Dark treatment as in (a). Exogenous oxidized thioredoxin was added where indicated by the arrow (▫———▫).

antibody preparation against *E. coli* thioredoxin [24] has been used to inhibit specifically the dark modulation of NADP-dependent malate dehydrogenase and glucose-6-phosphate dehydrogenase (Fig. 7) as well as the dithiothreitol-dependent activation of NADP-dependent malate dehydrogenase. In the latter system, the rate of inhibition was shown to be related to the amount of added antibody in a linear manner until saturation was obtained.

In conclusion, it can be assumed that the thioredoxin-catalyzed change of enzyme activities is reversible, depending on the redox state of the environment. Under dark conditions, i.e., when no electron source (light or dithiothreitol) is provided or when oxidants such as oxidized glutathione or dehydroascorbate [13] are added, the reverse reaction (i.e., SH-group oxidation) is favored. The oxidant functioning in vivo has not yet been identified, though it seems plausible that O_2 may be involved in the oxidation of the dithiol [17]. In the in vitro system reoxidation of thioredoxin seems to be hindered due to dilution thus becoming the rate-limiting step in dark modulation.

Since it has been shown previously that thioredoxin catalyzes the dithiothreitol-dependent modulation of most light-modulated enzymes, it is likely that thioredoxin functions as the dark factor in those cases as well.

Acknowledgements

This research was supported by a grant from US National Science Foundation (PCM-7926167). R.S. was a NATO Fellow on leave from the University of Bayreuth. We thank A.R. Ashton for many suggestions and useful discussion, A. Holmgren for providing antibody against *E. coli* thioredoxin, L. Sykora and staff at the University of Illinois Chicago Circle Greenhouse for growing the pea plants

and H.-J. Lah, S. Shah and M. Zbyszewski for preparing chloroplasts.

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