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Regulation of corn leaf NADP-malate dehydrogenase light-activation by the photosynthetic electron flow. Effect of photoinhibition studied in a reconstituted system

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The light-activation of the chloroplastic enzyme NADP-malate dehydrogenase is totally inhibited when chloroplasts are preilluminated with high light. The mechanism of this photoinhibition has been studied in a reconstituted chloroplast system composed of isolated thylakoids and the purified proteins of the ferredoxin-thioredoxin light activation system, by examinating the reduction state of the disulfide bridges located on the different proteins of the system and considered to be involved in the light activation process. The results indicate that, when the reduction of S-S groups on thioredoxin and on NADP-MDH is only partially decreased, NADP-MDH is totally inactive. A reduction vs. activity curve shows that the activity of this last enzyme strongly depends on its reduction state, more than 50% reduction being required for activity to appear. The physiological significance of these observations is discussed.

Introduction

Several chloroplastic enzymes are known to be activated through reduction by the photosynthetic electron transfer chain via the ferredoxin-thioredoxin system [1]. Compared to the requirement for reducing power by the Calvin cycle, this process requires only a small input of electrons [2]. It is surprising, then, that activation of these enzymes has been found to be very sensitive to preillumination with high light [3,4]. Photoinhibition by high light is considered to result mainly from damage to the photosynthetic electron transfer chain at the level of photosystem II reaction centers [5,6]. This damage causes only a partial inhibition of the open-chain electron transfer reactions [5]. Nevertheless, a strong decrease in the activities of several light-activated enzymes has been observed in crude extracts from high-light-pretreated leaves [3] and an inhibition of the activation of NADP-dependent malate dehydrogenase (NADP-MDH) upon high-light preillumination of intact chloroplasts has been reported [4]. These observations suggested that the modulation of NADP-MDH light-activation through photoinhibition could be an interesting experimental approach to study the regulation of the light-activation of NADP-MDH by the photosynthetic reducing power. In the present work, we applied this approach to a reconstituted light-activation system composed of washed isolated thylakoids, all the purified proteins of the ferredoxin-thioredoxin system and the light-activated target enzyme corn leaf NADP-MDH.

Material and Methods

Materials

Spinach (*Spinacia oleracea* L.) was purchased from the local market. Corn (*Zea mays* L., var. M64Ax-W182E) and peas (*Pisum sativum* L. var. Merveille de Kelvedon) were grown in the greenhouse as described previously [7].

Methods

Pea thylakoids were prepared as described in Ref. 8, i.e. washed in the last step with a hypotonic 50 mM Hepes pH 7.9 buffer containing 1 mM EDTA and 5 mM NaCl in order to eliminate membrane-bound thioredoxins, and finally suspended in a 50 mM Hepes

Abbreviations: Chl, chlorophyll; DPIP, 2,6-dichlorophenol indophenol; DTT, dithiothreitol; NADP-MDH, NADP-malate dehydrogenase; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)amino methane; FTR, ferredoxin-thioredoxin reductase.

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buffer pH 7.9, 330 mM sorbitol, 1 mM $MgCl_2$, at a chlorophyll concentration of ca. 2 mg/ml. Chlorophyll was determined as in Ref. 9.

The different proteins of the reconstituted system were prepared by already published procedures. Ferredoxin [10], thioredoxin m [11] and ferredoxin-thioredoxin reductase [12] were isolated from spinach leaves, while NADP-MDH [13,14] was purified from corn leaves. Additional HPLC steps were used to purify thioredoxin and NADP-MDH to homogeneity: a TSK SW 3000 (Pharmacia) size-exclusion step was used for the thioredoxin and a mono Q anion-exchange step for the NADP-MDH. Different plant sources were used in order to obtain good protein yields. It has been shown previously that the efficiency of the reconstituted system was not affected by this heterogeneity, as long as higher plant enzymes were used [7].

For the photoinhibition pretreatment, thylakoids were diluted to 300 μg Chl/ml in Eppendorf tubes and preilluminated with white light at a photon flux density of 4000 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ for different lengths of time (1 to 8 min). Preliminary experiments showed that the photoinhibitory effect did not depend on the presence of the proteins of the ferredoxin-thioredoxin system during the pretreatment. Control thylakoids were preincubated in the dark for the same time.

NADP-MDH light activation was carried out by adding all of the proteins of the light-activation system to the pretreated thylakoids, at the following, previously optimized, concentrations [8]: 10 μM ferredoxin, 2 μM ferredoxin thioredoxin reductase, 10 μM thioredoxin m, and 5 μM (10 μM subunits) NADP-MDH. Each 100 μl sample contained 20 μg chlorophyll. Samples were incubated under moderate light (400 μmol quanta $\cdot m^{-2} \cdot s^{-1}$) which was sufficient to saturate enzyme light activation [2] without producing photoinhibitory effects. After the activation period, a 10 μl aliquot was removed to measure the NADP-MDH activity. The remaining sample was used for thiol derivatization. To this sample [^{14}C]iodoacetamide was added to a final concentration of 3 mM (148 kBq/μmol, dissolved in 100 μM Tris-HCl, pH 8) and the reagent was allowed to react for 1 min in the light followed by 14 min in the dark. The thylakoids were removed by centrifugation and the supernatant was used for thiol quantitation.

NADP-MDH activity was measured spectrophotometrically by the decrease in absorbance at 340 nm, as described previously [15]. One unit of enzymatic activity represents 1 μmol NADPH oxidized per min.

Total thiol quantitation was realized using the supernatant of the spinned light-activation medium. The proteins present were precipitated for 2 h on ice with 10% TCA and centrifuged. The pellet was washed twice with TCA, then dissolved in 0.3 ml of 100 mM Tris-HCl buffer (pH 8), SDS 2% and counted in a scintillation counter in the presence of 3 ml of Instagel scintillation cocktail. To study the reduction state of the individual proteins of the system, the supernatant of the centrifuged light-activation medium was applied to a TSK 3000 SW size-exclusion HPLC column and eluted with a 30 mM Tris buffer (pH 7.9) containing 300 mM NaCl, as described [16]. This procedure allowed a quantitative recovery of each of the derivatized proteins. After separation, the elution volume of each of the proteins was measured, and an aliquot was counted in a scintillation counter.

In some experiments, NADP reduction was also measured. In this case, various concentrations of NADP were added to the reaction mixture. After exposure to light, the sample was diluted to 1 ml with cold 100 mM Tris-HCl buffer, thylakoids were removed by a short centrifugation in an Eppendorf microfuge and the NADPH formed was measured by its absorption at 340 nm. The addition of ferredoxin-NADP reductase to the reaction medium was not necessary, as the thylakoid-bound enzyme was already saturating.

Results

When studied in the reconstituted system, NADP-MDH light activation exhibited a high sensitivity to photoinhibitory treatments. After an 8 min preillumination of the complete light-activation medium with high light, NADP-MDH activation was totally abolished, while in parallel assays NADP photoreduction was inhibited by only 50% (Table I). Both processes could be restored to their original level by adding DPIP + ascorbate, indicating that photosystem II activity was the only limiting factor in the system. Restoration of the activity by the addition of either fresh thylakoids or DTT to the preilluminated light-activation mixture (Fig.

TABLE I

Compared photoinhibition of NADP photoreduction and NADP-MDH light activation in a reconstituted system

The reaction media (containing either 10 μM ferredoxin, or 10 μM ferredoxin + 2 μM FTR + 10 μM thioredoxin) were pretreated for 8 min with strong light (4000 μmol quanta $\cdot m^{-2} \cdot s^{-1}$). Then the light was decreased to 1500 μmol quanta $\cdot m^{-2} \cdot s^{-1}$, and the reaction was started by adding either NADP (4 mM final concentration) or MDH (0.5 μM in subunit concentration). NADP reduction or MDH light-activation was measured after 5 min. Control samples were preincubated for 8 min in the dark. Where indicated, 10 mM ascorbate and 2 mM DPIP were added after the preillumination period as electron donors to photosystem I.

	NADP reduced μmol/mg Chl per h	MDH activated U/mg Chl per h
Control	96	110
Photoinhibited	45	3
Photoinhibited + DPIP + ascorbate	85	120

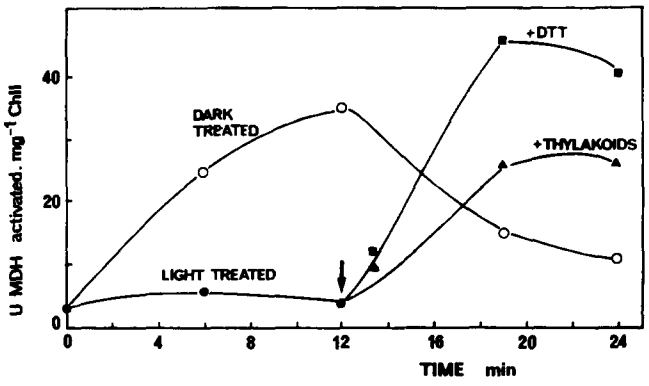

Fig. 1. Time-course of NADP-MDH light-activation after preillumination of the complete activation medium with high light (4000 μmol quanta·m^{-2}·s^{-1}) for 8 min. For the dark treatment, the medium was maintained 8 min in the dark. The activation was performed under a saturating light intensity (1500 μmol quanta·m^{-2}·s^{-1}). The arrow indicates the time of addition of either 10 mM dithiothreitol, or untreated thylakoids (20 μg Chl).

1) further indicated that the added proteins of the ferredoxin-thioredoxin system were not directly damaged by the high-light treatment. In these series of experiments, the light used for activation was relatively high (1500 μmol quanta · m^{-2} · s^{-1}), in order to saturate NADP photoreduction. Under these conditions, the MDH activity of the control sample decreased after 15 min of illumination, as a result of photoinhibition (Fig. 1). In further experiments, where NADP photoreduction was not measured, the activation-light intensity was decreased to 400 μmol quanta · m^{-2} · s^{-1} to avoid this drop in activity. The extent of photoinhibition was the same whether the proteins of the ferredoxin-thioredoxin light activation system were added before or after the preillumination (data not shown). Then, the pretreatment of thylakoids alone was adopted for further studies.

NADP-MDH light activation is the final reducing step of a disulfide reduction chain where the dithiol generated on ferredoxin-thioredoxin reductase by reduced ferredoxin reduces the disulfide bridge of thioredoxin m which in turn reduces NADP-MDH [8,14]. The lack of activation of NADP-MDH could originate in a poor efficiency of the previous reduction steps, if the proteins of the ferredoxin-thioredoxin system are much less readily reducible by ferredoxin than NADP is. To check this hypothesis, thioredoxin plus FTR photoreduction was measured in the presence of various concentrations of thioredoxin and NADP and in the absence of NADP-MDH. Photoreduction of the proteins was determined by derivatizing their SH groups with ^{14}C-labelled iodoacetate.

No attempt was made at this stage to separate thioredoxin from FTR after derivatization, since the FTR concentration (2 μM) used was low relative to that of thioredoxin and thus could account for no more than 25% of the total SH at the lowest thioredoxin concentration (8 μM), and much less at higher thioredoxin concentrations. Furthermore, a preliminary experiment run with FTR alone (without thioredoxin) showed that FTR reduction was decreased very similarly to thioredoxin reduction, i.e. only slightly (data not shown). Consequently, the small changes in its reduction state could not be measured with accuracy in the presence of thioredoxin. For all these reasons, the results presented in Fig. 2 can be considered to reflect mainly the reduction of thioredoxin. They show that the competition between NADP and thioredoxin reduction pathways is weak, with a K_i for NADP of 2.3 mM relative to thioredoxin reduction. It could be observed only in conditions of limiting light: under saturating light (and with untreated thylakoids), the photosynthetic electron transfer was sufficient to ensure both reductions at a maximal rate, and no inhibition of thioredoxin reduction by NADP could be obtained (data not shown). Then the efficiency of the electron transfer leading to thioredoxin reduction was apparently very similar to the one leading to NADP reduction.

This point was further confirmed by looking at the reduction rates of thioredoxin plus FTR (i.e. mainly thioredoxin) as a function of the length of the photoinhibitory treatment, in a reaction mixture from which NADP-MDH was omitted, and comparing them to the

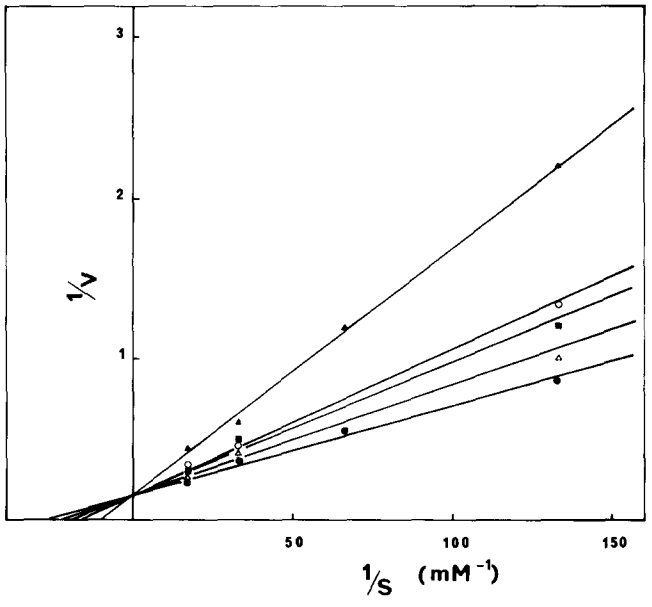

Fig. 2. Inhibition of the reduction of thioredoxin in the reconstituted system in the presence of different NADP concentrations under limiting light (400 μmol quanta·m^{-2}·sec^{-1}). NADP concentrations used (mM): ●, 0; △, 0.6, ■, 1.5; ○, 3; ▲, 7.5. The Lineweaver-Burk type representation allows the calculation of a K_i for NADP of 2.3 mM. $1/V$ is expressed in arbitrary units, based on the amount of [^{14}C]iodoacetate incorporated in the SH groups of thioredoxins and FTR after a 30 sec illumination. $1/S$ represents thioredoxin concentrations (mM^{-1}).

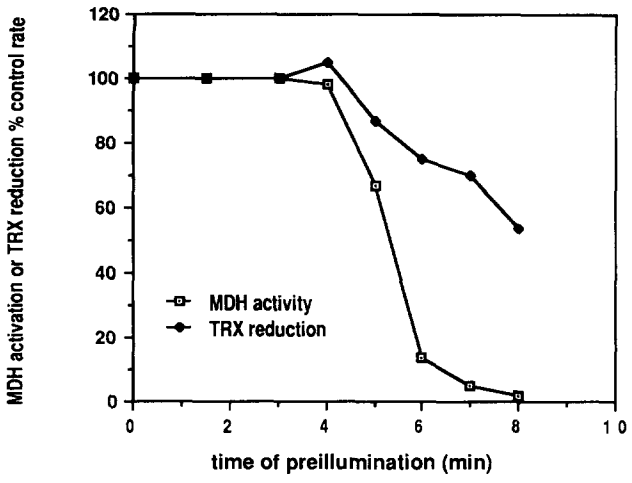

Fig. 3. Variations in the rates of either thioredoxin reduction or NADP-MDH light-activation in the reconstituted system as a function of the length of the photoinhibitory treatment. The assays were run on parallel samples, thioredoxin reduction being measured in the absence of NADP-MDH. In order to measure linear rates with sufficient accuracy, thioredoxin reduction was measured after a 30 s activation period, and MDH activity after a 4 min activation period. The results are expressed as the % of the control value obtained with dark-pretreated samples.

NADP-MDH activation rates obtained with a complete reaction medium. Under conditions where NADP-MDH light-activation was totally inhibited, the rate of reduction of thioredoxin was decreased by only about 30–50% (Fig. 3) i.e. approximately to the same extent as NADP photoreduction (see Table I). When thioredoxin and NADP-MDH were both omitted from the reaction medium (only ferredoxin and FTR added after the photoinhibition pretreatment) the FTR reduction rate was decreased to a similar extent (50% after an 8 min treatment, result not shown). Clearly, the NADP and thioredoxin photoreduction pathways exhibited very similar sensitivities with respect to photoinhibition. Consequently, the total inhibition of MDH light activation after a high-light treatment could not be ascribed to the complete inhibition of thioredoxin reduction.

The results in Fig. 3 show that a preillumination treatment sufficient to completely suppress NADP-MDH light activation lowered the thioredoxin reduction rate by only about 30%. This observation was very similar to the results reported by Rébeillé and Hatch [17] for NADP-MDH activation rates by partially reduced thioredoxin, when thioredoxin reduction state was modulated by different ratios of reduced to oxidized dithiothreitol. The authors interpreted this result as a requirement for a high ratio of reduced to oxidized thioredoxin in order to reduce NADP-MDH, due to a more electronegative oxido-reduction potential for MDH than for thioredoxins. To further investigate if this interpretation could apply also to our experiments, we needed to quantitate separately the reduction state of NADP-MDH and of thioredoxins in our more complex reconstituted system.

We found that we could obtain a quantitative recovery of each of the labelled proteins of the system by using HPLC size-exclusion chromatography [16]. Optimal separation required that iodoacetamide instead of iodoacetate be used to derivatize the SH groups and that 0.3 M NaCl be included in the filtration buffer. With iodoacetate, some interaction with the matrix was observed, and without NaCl, FTR and ferredoxin comigrated as a complex with an apparent molecular weight very similar to the one of NADP-MDH [16]. This ability of ferredoxin and FTR to form stable complexes has already been reported in previous studies [12,18].

The most striking result of this quantitation was that totally inactive NADP-MDH was not synonymous with a totally oxidized protein (Fig. 4). In fact, the graph showing the extent of reduction of NADP-MDH as a function of the length of the photoinhibitory treatment was very similar to the one obtained for thioredoxin reduction rates in Fig. 3. The graph showing thioredoxin reduction state was somewhat different. This was not due to the presence of MDH in the reaction medium, but simply to the fact that in Fig. 4 the determination of thioredoxin reduction was done after a longer period of time than in Fig. 3, and thus reflected steady-state reduction levels rather than reduction rates. The activation level of NADP-MDH was much more affected by the preillumination than its reduction state, the enzyme being totally inactive when its reduction was decreased by only about 50%. This result indicated that the activ-

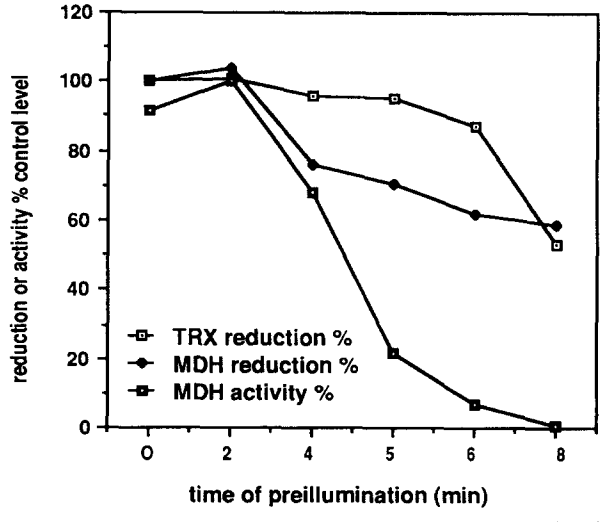

Fig. 4. Reduction levels of thioredoxin and NADP-MDH and activation state of NADP-MDH as a function of the length of the photoinhibitory pretreatment. All the data were obtained after a 4 min activation period. The proteins of the ferredoxin-thioredoxin light-activation system were separated after derivatization of their SH groups. The results are expressed as the % of the control value obtained with dark-pretreated samples.

ity of the enzyme was mainly dependent on its own reduction state. On the fully reduced and activated enzyme, 2 SH groups could be quantitated per subunit. The same number of SH groups could be obtained when NADP-MDH was directly activated by DTT, without thioredoxin, which is possible in the presence of high Tris or high NaCl concentrations [14,19], and a similar difference between the reduction and the activation curves was obtained in this case (Fig. 5), the half-reduced enzyme being totally inactive.

The results from different experiments (either direct activation by DTT in the presence of high Tris, or light-dependent activation in the complete reconstituted system after different photoinhibition treatments, and at various times of activation) were plotted together to determine if there was a correlation between the activity of MDH and its reduction state. All the results fitted a single curve which appeared to have a sigmoïdal shape (Fig. 6). This curve was somewhat different from the one obtained by Scheibe [20] for DTT-activated pea NADP-MDH which coincided with the theoretical probability curve of a reduced tetramer being the active form of the enzyme.

Discussion

The study of the effect of photoinhibition on the light-activation of NADP-MDH in a reconstituted system confirms that despite its low energy requirement this process is very sensitive to the damage caused to the photosynthetic electron transfer chain by high light (Table I, Fig. 1). Previous studies done either with whole leaves [3] or with intact chloroplasts [4] suggested that the sensitivity of NADP-MDH activation to high light was at least partially due to a competition for

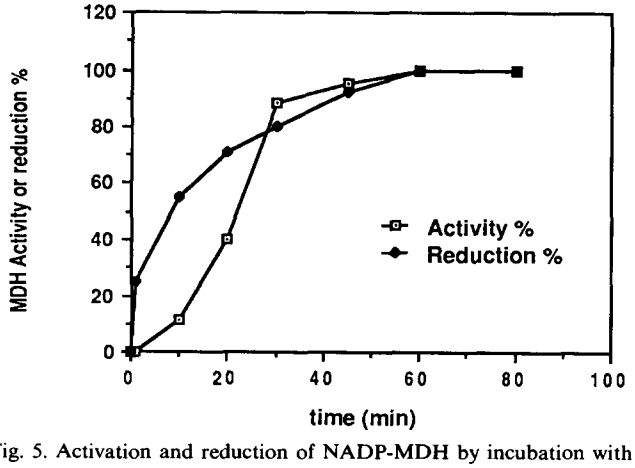

Fig. 5. Activation and reduction of NADP-MDH by incubation with 10 mM DTT in the presence of 0.7 M Tris buffer (pH 7.9). The results are expressed as % of the maximal value which corresponded to the reduction of two SH groups per subunit enzyme. In a total volume of 150 µl, each sample contained 0.7 M Tris-HCl buffer, 10 mM DTT and 1 nmol subunit NADP-MDH.

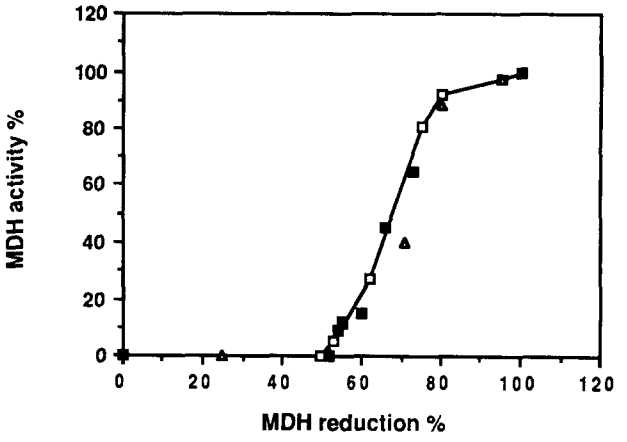

Fig. 6. Relationships between the extent of reduction and activation of NADP-MDH, expressed as the % of maximal values (corresponding to the reduction of 2 SH groups per subunit). The results are taken from different experiments: either direct activation by DTT (■), or activation by light in a reconstituted system (□,△) after different lengths of photoinhibitory treatments followed by either 2 or 4 min MDH light-activation.

electrons with CO_2 fixation [4]. However, no definite conclusion about the precise step(s) affected by photoinhibition could be drawn, due to the complexity of the system. Particularly, it was difficult to decide whether this competition took place at the level of reduced ferredoxin, the common electron donor for NADP reduction and enzyme activation, or was caused by increased levels of unreduced NADP which is known to inhibit directly NADP-MDH activation [21,22]. In the reconstituted system, the hypothesis of a competition for reduced ferredoxin can be checked directly, by measuring the effect of NADP on the reduction of thioredoxin in the absence of NADP-MDH (Fig. 2). The results clearly show that the efficiency of thioredoxin reduction is very close to the one of NADP reduction.

Under saturating light, both pathways can occur at optimal rates, without any competition. Under limiting light, the competition is weak, the K_i for NADP, determined with various concentrations of thioredoxin and of NADP, being higher than the mean intrachloroplastic concentration of this nucleotide [23]. The effect of NADP on NADP-MDH light-activation can thus be mainly ascribed to a direct effect on the target enzyme rather than to a competition for electrons at the level of ferredoxin.

The use of the reconstituted system in the presence of NADP-MDH reveals that even in the absence of NADP, the activation of the enzyme is fully photoinhibited, suggesting that one of the steps in the oxido-reduction chain leading to the activation of NADP-MDH is particularly affected by high light. This step is neither thioredoxin reduction, which is only partially inhibited (Fig. 3), nor NADP-MDH reduction (Fig. 4) which is

affected to the same extent as thioredoxin reduction. It seems, rather, that the generation of only partially reduced NADP-MDH results in a completely inactive enzyme, i.e. only a completely reduced enzyme molecule would be active.

Studies by Scheibe [24] on NADP-MDH from pea leaves indicated that the enzyme was composed of four identical, or almost identical subunits, each of them bearing a DTT-reducible disulfide bridge. When pea enzyme was activated directly by DTT (without thioredoxin) in the presence of a high NaCl concentration, the reduction vs. activity curve coincided with the probability curve of getting four identical subunits reduced at a time in a random distribution [20].

Corn NADP-MDH is considered as a dimer [13,16,25]. The number of reducible disulfides which was reported up to now for this enzyme is variable: one [13,14], two [26] or three [25] dithiols could be quantitated per subunit after reduction. Only one of them, reducible by DTT, but not by mercaptoethanol seems to be involved in the activation of the enzyme [25]. In our hands, reduction either by DTT or by light-dependent electron transfer yielded maximally two SH groups per subunit when thiol derivatization was done with [^{14}C]iodoacetate. Two additional thiols appeared upon denaturation [14]. Sequencing of the radioactive peptides obtained after activation of the enzyme either by DTT or by light, followed by thiol derivatization with [^{14}C]iodoacetate and tryptic digestion showed that the major part of the radioactivity was located on N-terminal cysteines 10 and 15 which are linked in a disulfide bridge in the inactive molecule [14]. Other radioactive peptides were identified, but they were only weakly labelled, suggesting that the corresponding cysteines were poorly accessible to the derivatizing reagent. Then we can assume that despite the presence of 8 cysteines in the amino-acid sequence of the MDH monomer [27], only the two cysteines linked in a disulfide bridge at the N-terminus of each subunit can be efficiently derivatized by [^{14}C]iodoacetate upon reduction. The reduction vs. activation curve (Fig. 6) can thus be considered to represent the extent of reduction of this N-terminal disulfide bridge which is essential for the appearance of the activity of the enzyme. We have already observed during our sequencing work that when NADP-MDH was only partially reduced, the labelling of its N-terminal cysteines was decreased to the same extent as the labelling of the whole molecule (unpublished data), thus ruling out the possibility that the absence of activity upon partial reduction was due to the reduction of other disulfide bridges than the N-terminal one. Then, the only likely interpretation of the data presented in Fig. 6 is that all the subunits of the enzyme must be reduced for the activity to appear, the reduced monomer being not an active form. It is worth to notice that the results obtained either in light activation or in DTT activation (with or without thioredoxin) fit a single curve: this indicates that activation by DTT produces similar modifications on NADP-MDH as light activation does, and is therefore a valuable model. Nevertheless, the experimental curve in Fig. 6 does not fit the theoretical probability curves for getting either two or four reduced subunits at a time in a random distribution (the theoretical curves can be found in the paper by Scheibe [20]): it falls between those two curves and its sigmoidal shape rather suggests cooperativity effects, a partial reduction of the molecule facilitating its full activation.

This specific property of NADP-MDH would provide a very efficient means of regulating the activity of this enzyme which is involved in the export of the reducing power from the chloroplast to the cytosol by providing the malate for the malate/oxaloacetate shuttle of the chloroplast envelope. Because the partially reduced enzyme is inactive, NADP-MDH can be fully active only under conditions where the reduction state inside the chloroplast is maximal, thus avoiding the efflux of reducing equivalents which are needed for the Calvin cycle. A dependence of the NADP-MDH activity in intact chloroplasts on a high reduction state in the stroma has already been demonstrated in intact chloroplasts [28,29]. The photoinhibition effect provides an additional demonstration of this regulation, showing that a partial decrease in the electron transfer efficiency can result in a complete inhibition of NADP-MDH activity, even when the enzyme molecule is still partially reduced.

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