On the Mechanism of Activation by Light of the NADP-dependent Malate Dehydrogenase in Spinach Chloroplasts

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ABSTRACT

With intact spinach (Spinacia oleracea L. cv. Vital R) chloroplasts, the activity of the NADP-dependent malate dehydrogenase after activation by light was 30 micromoles of malate formed per milligram of chlorophyll per hour; an identical rate of O₂ evolution was obtained upon oxaloacetate reduction by the intact plastids. However, when the activity of NADP-dependent malate dehydrogenase was measured subsequently to maximal activation of the enzyme by dithiothreitol (DTT) an average rate of 113 micromoles per milligram of chlorophyll per hour was obtained. When membranes and stroma were separated after osmotic disruption of the chloroplasts, 28% of NADP-dependent malate dehydrogenase activity inducible by DTT was found with the membranes and 72% was found in the stromal fraction. The membrane-associated portion of the enzyme corresponds well with the activity achieved after activation by light. About 64% of an activator system was found to be associated also with the membrane fraction. Washing the membranes with buffer removed more activator than enzyme. However, both were removed almost completely by ethylenediaminetetraacetate. It was concluded that both a portion of the enzyme and the total activator system are associated with the chloroplast membranes in vivo and that the activator is more loosely bound than the enzyme. A model describing the partial activation of chloroplastic NADP-dependent malate dehydrogenase by light and the total activation by DTT is presented.

The chloroplastic NADP-dependent MDH has been described as a soluble stromal enzyme which is activated in vivo by light (2, 12). In vitro light can be replaced by DTT (1, 15). This indicates that the activating reaction is a reductive one. It has been suggested that the reducing power is transferred from ferredoxin via ferredoxin-thioredoxin reductase and the activator thioredoxin to the enzyme which is dissolved in the stroma (17, see also 9). On the other hand, Anderson et al. (4) have presented evidence that activation of NADP-dependent MDH by light is initiated by an intramolecular thiol-disulfide exchange catalyzed by a membrane-bound light effect mediator (LEM, 3). This model transfers the place of MDH activation from the stroma to the chloroplast lamellae.

In both models one basic problem appears not to have been considered adequately: if the NADP-dependent MDH or the activating system were components of the stroma, the total stroma must be brought into close contact with the thylakoid surface in a very short time because activation by light is a rapid process (3). Since the stroma is a viscous solution in which the predominant fraction I protein may occur sometimes in a crystalline state (14), a fast circulation of the whole stroma is as difficult to imagine as is a rapid diffusion of single specialized polypeptides in the sticky mass.

This difficulty can be overcome by a model in which the activator or mediator and the enzyme are bound to or at least associated with the chloroplast lamellae. In this paper, evidence for such a model will be presented.

MATERIALS AND METHODS

Preparation of Isolated Chloroplasts and Chloroplast Fractions. Intact chloroplasts were isolated from spinach (Spinacia oleracea L. cv. Vital R) leaves (grown under controlled short day conditions) by a procedure similar to that of Jensen and Bassham (11) which has been described elsewhere (5).

Isolated and purified chloroplasts were osmotically disrupted by gently shaking the pelleted intact chloroplasts with sorbitol-free 0.01 M Tris-HCl buffer at pH 8.0 (containing 4 mM MgCl₂) for 5 min at 0°C. Where indicated, 1 mM EDTA was added to the disruption solution instead of MgCl₂. A stromal and a membrane fraction were separated from the resulting suspension by centrifugation at 12,500g (5 min). In some experiments the latter fraction was washed twice by resuspension with the same buffer and centrifugation as before. The volume of each fraction (stromal fraction, first and second wash solutions, and membrane fraction) was made up to that of the original disruption suspension by addition of the same sorbitol-free buffer.

Activation of NADP-dependent MDH. For activation by light intact chloroplasts equivalent to 40 to 50 μg of Chl were suspended in 1 ml of Hepes buffer C described by Jensen and Bassham (11). The continuously stirred suspension was illuminated with white light (200 w/cm²) in a water bath at 25°C.

For activation by DTT unfractionated broken chloroplasts or single or combined chloroplast fractions were incubated at 25°C in a solution containing 0.1 M Tris-HCl buffer (pH 8.0), 4 mM MgCl₂, and unless stated otherwise, 50 mM DTT.

Determination of Enzyme Activity. The activity of the NADP-dependent MDH was determined by OAA reduction using a slightly modified assay according to Hatch and Slack (8). Aliquots of chloroplast suspensions activated by light or of chloroplast fractions activated by DTT were assayed in 1 ml of hypotonic reaction mixture containing 100 mM (or 10 mM where indicated) Tris-HCl buffer (pH 8.0), 1 mM EDTA, 0.2 mM NADPH, 0.5 mM OAA. The reaction was started about 1 s after the withdrawal of the sample.

The following observations contribute to the question whether there is inactivation of the light-dependent enzyme activity in the dark after osmotic rupture in the assay.

a. A time course of an experiment using light for activation is given in Figure 1. When the curve obtained without OAA (control) is subtracted from the curves obtained with the complete assay the actual rate increased for the 1st min according to the proceeding osmotic disruption and was then constant for at least another 6 min (dotted lines).

b. In another experiment part of the illuminated chloroplast suspension was removed, transferred immediately into the 10-fold
ACTIVATION BY LIGHT OF NADP-DEPENDENT MDH

Figure 1. Time course of NADP-dependent MDH reaction. Complete assay contained 10 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 0.2 mM NADPH, 0.5 mM OAA. The reaction was started (arrow) by the addition of a suspension of illuminated (5 min) or darkened intact chloroplasts corresponding to 6 μg Chl.

RESULTS

Association of NADP-dependent MDH with Chloroplast Lamellae. Intact spinach chloroplasts were illuminated with white light (200 w/cm²) and then transferred to a hypotonic medium in a test tube. The activity of the NADP-dependent MDH was measured immediately after osmotic rupture of the chloroplasts. Average activities of about 30 μmol OAA reduced/mg Chl·h were obtained (Fig. 2 and Table II). When the chloroplasts were allowed to reduce OAA photosynthetically in the cell of an oxigraph, the rate of O₂ evolution was equivalent to that found for NADP-dependent MDH activity.

Upon activation of NADP-dependent MDH by 50 mM DTT instead of light, average rates of OAA reduction of 113 μmol/mg Chl·h were obtained (Table II). Since NADP-dependent MDH activities resulting from activation by light on the one hand and from incubation with DTT on the other were determined in identical assays, the higher activity induced by DTT cannot be ascribed to NADP-dependent MDH plus NADPH phosphatase action on NADPH. In addition no decrease of activity was observed when 10 mM NaF was added to the assay as a phosphatase inhibitor.

Therefore, it has to be assumed that chloroplastic NADP-dependent MDH is only partly, i.e. 25 to 30%, activated by light. If this were so, it is conceivable that this light-activated portion comprises predominantly the NADP-dependent MDH molecules which are close to or associated with the chloroplast lamellae providing the reducing power for the activation. To examine this hypothesis, isolated chloroplasts were separated by centrifugation into a membrane and a stromal fraction after osmotic disruption. The activation of the NADP-dependent MDH by DTT was followed in both fractions and in the combined system. The results given in Figure 3 show that maximal activities were not obtained simultaneously in the individual assays. The combined system as well as the membrane fraction alone reached their maximal activities after 30 to 40 min whereas the activity in the stromal fraction increased up to about 5 h. The data further show that in systems which contain membranes an inactivation process takes place with time. Such a process has been described by Vidal et al. (16).

Most important is the fact that on the average 28% of the total DTT-induced enzyme activity was found with the membrane fraction. This portion corresponds with the portion of the enzyme which could be activated by light (Table II). Since, after osmotic rupture of the chloroplasts, the volume of the membranes usually was less than 5% (as determined with the hematocrit technique) of that of the stromal fraction, the NADP-dependent MDH activity in the membrane fraction cannot be ascribed to a heavy contamination of the chloroplast lamellae by stroma compounds.

Association of Activating System with Chloroplast Lamellae. Purified chloroplastic NADP-dependent MDH cannot be activated solely by DTT (17). It is known that in the activation by light as well as by DTT an activator is involved which has been identified as thioredoxin (6, 17). The second factor, designated as ferredoxin-thioredoxin reductase (17), seems not to be required for activation by DTT.

If an activator compound is necessarily involved in the activating process, the membrane fraction must contain both NADP-dependent MDH and the activator. Following the kinetics, maximal activation rates were observed after a few minutes' incubation of the chloroplast fractions with DTT. It is obvious from Figure 3 that at this time the amounts of enzyme were not limiting the activation rates. Therefore, the maximal rates should provide a

<table>
<thead>
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<th>Conditions as described in the legend of Figure 1.</th>
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<tbody>
<tr>
<td><strong>Illumination Period</strong></td>
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<tr>
<td>----------------------------</td>
</tr>
<tr>
<td><strong>min</strong></td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>10</td>
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Figure 2. OAA oxidation by NADP-dependent chloroplastic MDH. OAA oxidation, expressed as NADPH oxidation (ΔOD 340 nm) was measured spectrophotometrically as described in Calculation of Results. The enzymatic activity of chloroplasts was measured in the dark.
FIG. 2. Activation and inactivation of NADP-dependent MDH in intact chloroplasts by light and darkness, respectively. Intact chloroplasts (equivalent to a Chl concentration of 54 μg/ml) were suspended in 0.05 m Hepes buffer (pH 7.6, containing 2 mM NaNO₃, 2 mM sodium ascorbate, 2 mM EDTA, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM K₂HPO₄, 7.7 mM NaHCO₃, 0.33 mM sorbitol) and illuminated with saturating white light (200 W/m²).

The temperature was maintained at 25 C. As indicated in the diagram, samples of 100 μl were withdrawn and the activity of NADP-dependent MDH was measured immediately in the same hypotonic medium as described in the legend of Figure 1.

Table II. Distribution of DTT-induced NADP-dependent MDH Activity and of the Activating System in the Chloroplast Fractions and Comparison of the Enzyme Activity Obtained after Activation by Light and by DTT

<table>
<thead>
<tr>
<th>Fraction Used</th>
<th>Combined System</th>
<th>Stomatal Fraction</th>
<th>Membrane Fraction</th>
<th>Intact Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation by</td>
<td>DTT (50 μM)</td>
<td>Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme activity* (μmol malate/mg Chl h⁻¹)</td>
<td>113</td>
<td>81</td>
<td>32</td>
<td>29.3</td>
</tr>
<tr>
<td>% of enzyme activity</td>
<td>100</td>
<td>71.7</td>
<td>28.3</td>
<td>26</td>
</tr>
<tr>
<td>Activation velocity* (spec. act./10 min)</td>
<td>38</td>
<td>7</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>DTT concentration for half-maximal activity (μM)</td>
<td>12</td>
<td>5</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Activator distribution (%)</td>
<td>36</td>
<td>64</td>
<td></td>
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</table>

* Average of 10 experiments (representing the maximum activities achieved with experiments like that given in Fig. 2 or 3, respectively).

* Average of 5 experiments (using the linear activation rates as indicated in Fig. 3).

* Data calculated from Fig. 4.

measure of the efficiency of the activator in the individual chloroplast fractions. If the activator distributes equally in the solution its efficiency would correspond to its concentration. Since this is probably not so with the membrane fraction (see below), only the activator efficiency (expressed as Δ activity per 10 min) in the membrane and in the stromal fractions could be compared. The rates given in Figure 3 show a significantly better activator efficiency in the membrane fraction than in the stroma solution. This higher efficiency could be due to a higher content of activator in the membrane fraction or to a concentration effect when both the activator and the enzyme are associated with the chloroplast lamellae.

In order to determine the (relative) activator concentrations in the chloroplast fractions, DTT was regarded as a substrate of the activator, and the DTT saturation of the maximum activation rate was titrated in each fraction and in the combined system (Fig. 4). Half-saturation of the membrane fraction was achieved with 9 mM DTT and that of the stroma solution was obtained with 5 mM DTT. Given that the interactions between DTT and the activator are identical in each fraction, this indicated that the membrane fraction contained about twice as much activator as did the stromal fraction.

The fact that both NADP-dependent MDH and activator are fixed on the chloroplast lamellae could be interpreted by a variety of explanations ranging from a strong multi-enzyme-like complex of all components to unspecific protein-binding interactions. In the latter case enzyme and activator should be easily extractable from the membranes either by a stepwise washing procedure or by using complexing reagents such as EDTA.

In the first type of experiments the chloroplast membranes were stirred with hypotonic Tris-HCl buffer (0.01 M, pH 8) and subsequently centrifuged at 12,500g. The supernatant solution was removed and the washing procedure was repeated a second time. In each fraction both NADP-dependent MDH activity and activator efficiency were determined by means of activation kinetics (Fig. 5). Whereas in the stromal fraction the ratio of activator efficiency (activity/10 min): enzyme activity (μmol OAA reduced/mg Chl h⁻¹) was <1 (0.6) this ratio was >1 (2.0; 1.9; 1.5) with both wash solutions and the remaining membrane fraction, respectively. Therefore it was concluded that both the enzyme and the activator can be removed stepwise from the membranes, but that the activator is more easily extractable than the enzyme. This means that the enzyme as well as the activator are peripheral proteins rather than integral.

In a second type of experiment the intact chloroplasts were osmotically disrupted by the same Tris-HCl buffer as before, but 1 mM EDTA was added. In a stromal fraction obtained after such a treatment 95% of the activator efficiency and 90% of the enzyme was found (Fig. 6).

The high extractability of enzyme and activating system with a complexing reagent suggests also a weak association of both types of molecules with the chloroplast lamellae.

FIG. 3. Time course of activation of NADP-dependent MDH by DTT. Incubation mixture contained in a total volume of 1 ml 0.1 M Tris-HCl buffer, 4 mM MgCl₂, 50 mM DTT, 100 μl chloroplast fraction. Temperature was 25 C. Activity of the enzyme at the times indicated in the diagram was determined as described under "Materials and Methods."
DISCUSSION

When the light-induced activity of the NADP-dependent MDH (29 μmol OAA reduced/mg Chl-h) is compared with that obtained after activation with DTT (113 μmol OAA reduced/mg Chl-h) it is obvious that only about one-quarter of the total enzyme capacity of the chloroplast has been activated in vivo. This finding of a partial light activation is corroborated by the following results.

a. About one-quarter of the total enzyme found in the chloroplast after DTT activation was associated with the chloroplast lamellae and could more or less be removed by repeated washing of the membranes with buffer or by treatment with EDTA.

b. About two-thirds of the activator could be pelleted together with the membranes, a portion which might be sufficient for fast light activation of the membrane-bound part of the enzyme. However, it should be borne in mind that the association of the activator with the lamellae in the washing experiments was found to be looser than that of the enzyme. Therefore, it could be assumed that in the intact chloroplast more than two-thirds of the activator is associated with the membranes. This hypothesis could provide an explanation for the relatively slow activation by DTT in the membrane fraction (about 40 min, Fig. 3) as compared with the fast light activation (45–60 s, Fig. 2). Furthermore, the change in proton and Mg concentrations initiated by the illumination of the intact chloroplasts will occur more rapidly in the microenvironment of the thylakoids than in the stroma space: alkalinization and an increased Mg concentration are working toward rapid activation of the NADP-dependent MDH (16).

c. A significantly lower activity of NADP-dependent MDH after activation by light than by DTT was recently described also by Gupta and Anderson (7) for Kalanchoë leaves.

From these results the model presented in Figure 7 has been developed which describes the partial light activation of the NADP-dependent MDH in vivo (A) and the total activation of the enzyme by DTT in vitro (B). In the intact plastid the activator and about 28% of the enzyme were found to be associated with the membranes. This arrangement would allow a fast activation of the enzyme by reducing equivalents provided by reduced ferredoxin. In vitro, when part of the activator has been removed from the membranes, activation of the enzyme in the stromal and in the membrane fractions can be achieved by incubation with DTT.
Since in the latter fraction 72% of the total enzyme has to be activated by about one-third of the activator, the activation of the enzyme in the stromal fraction takes much more time than does that in the membrane fraction. In addition, in this fraction, enzyme and activator are assembled by the membranes, and the resulting concentration effect should cause an enhanced activation.

Preliminary results with chloroplasts from old spinach leaves or from spinach grown under not controlled conditions show that the portion of the activator which was found with the membranes may vary. However, the fact that only 26% of the NADP-dependent MDH could be activated by light suggests that there is indeed no operative diffusion of the enzyme molecules within the stroma area.

The model also explains some seemingly contradictory findings in the literature. The membrane-bound LEM described by Anderson and Avron (3) could be interpreted in terms of the activator which is associated with the membranes in the intact chloroplast. On the other hand the extractability of the activator could explain the fact that Wolosiuk et al. (17) and Jacquot et al. (9) could isolate an activating protein factor from a soluble extract of the leaf or intact spinach chloroplasts, respectively (6). In addition, Buchanan et al. (6) and Jacquot et al. (10) reported the presence of different thioredoxins within the chloroplast. The latter authors even isolated two different types of the so-called chloroplastic thioredoxin m which activate the NADP-dependent MDH (10). The data available up to now, however, do not allow us to draw conclusions whether these two types correspond to the associated and the solubilized type of the activator found in our experiments.

The activity of NADP-dependent MDH of the C4 plant Zea mays may be regulated in a similar manner, since a small protein factor involved in the activation of the enzyme by DTT has been isolated from this plant (13).

Finally the presence of membranes in an activation mixture containing DTT leads to an inactivation of NADP-dependent MDH which becomes more and more pronounced with time and depresses the maximum rates of the enzyme catalysis. It might be an expression of the inactivating system switching off the NADP-dependent MDH in the dark. In the stromal fraction alone this inactivation was never observed. Such an inactivation process has also been described by Vidal et al. (16) for Phaseolus and by Kagawa and Hatch (13) for Z. mays.

LITERATURE CITED