Interaction of Ferredoxin-NADP Oxidoreductase with the Thylakoid Membrane

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ABSTRACT

The binding of ferredoxin-NADP reductase to spinach chloroplast membranes was studied by washing the membranes with different media. Release of the enzyme from the thylakoids was greater in 0.75 millimolar EDTA but was not complete inasmuch as 20% of the activity remained membrane-bound after three washes.

A Scatchard plot of binding experiments suggests the presence of one type of binding site and a stoichiometry of 3 to 4 nanomoles of reductase per micromole of chlorophyll was calculated. Rebinding has a nonspecific requirement for cations. Their effectiveness increased with their valency. Rebinding of purified enzyme to depleted membranes resulted in a stimulation of its diaphorase activity.

It is suggested that binding of ferredoxin-NADP reductase to thylakoid membranes is dependent upon neutralization of negative charges.

Ferredoxin-NADP oxidoreductase (EC 1.18.1.2), the enzyme which catalyzes the final step in the photosynthetic NADP reduction in chloroplasts (21, 22), is located on the stroma surface of the thylakoid membrane.

The available information on the interaction of this FAD-containing protein with the membrane system is intriguing. Boger (6), in his work on transhydrogenases, classified this enzyme among the soluble reductases, inasmuch as it may be easily purified and remains stable in solution for several months. He also suggested that the reductase may be considered as an extrinsic protein, loosely attached to the thylakoids. However, at least to some extent, the reductase molecules are tightly bound to the membrane. This conclusion is based on the following observations: (a) the stroma-freed membranes are capable of carrying out in vitro NADP reduction, when supplied with ferredoxin (21); (b) fresh lysis of intact spinach chloroplasts in hypotonic media released less than 20% enzyme into the medium (7, 8, 23). Interestingly, the degree of solubilization is negligible in the eukaryotic alga Bumillieriopsis (6) and Chlorella (18) while almost complete in the cyanobacterium Nosococcus muscorum (6). By examining the kinetics of antibody inhibition in stacked and unstacked membrane preparations, Jennings et al. (17) concluded that the reductase is located outside the partition zones, presumably very near to the chloroplast coupling factor 1 (5). The nature of the forces involved in the protein-membrane interaction is also a matter of speculation. Treatment of chloroplasts with EDTA resulted in solubilization of 60 to 70% of the enzyme, suggesting that divalent cations should be necessary for binding (15, 25, 28). More significantly, the stimulation of diaphorase activity by synthetic polyamines further suggests the involvement of an electrostatic mechanism in the binding process (23).

The membrane-bound enzyme shows allosteric properties (10) and conformational changes are induced by light energization of the thylakoids (11, 12), suggesting a close interaction between the membrane and the enzyme.

In the present paper, we have dealt with the effects of EDTA and cations on the interaction between ferredoxin-NADP reductase and the chloroplast membrane, both during solubilization from and re-binding to the membrane. We discuss the importance of electrostatic phenomena on the attachment of extrinsic proteins to the membrane surface.

MATERIALS AND METHODS

Preparation of Chloroplasts. Intact chloroplasts were isolated from fresh spinach leaves (Spinacia oleracea) essentially as described by Bahr and Jensen (1) in an isolation medium of 50 mm Mes-NaOH (pH 6.8), 330 mm sorbitol, 5 mm MgCl2, 2 mm EDTA, 2 mm sodium ascorbate, 5 mm Pii, and 0.5 mm KH2PO4. The loosely packed surface of the pellet was rinsed away and the remaining pellet was suspended in isolation medium and centrifuged again at 1000g for 1 min. The percentage of intact chloroplasts measured by the ferricyanide-dependent O2 evolution (20) ranged from 75 to 85%. Total Chl was determined as described (27). Ferredoxin (9) and ferredoxin-NADP reductase (29) were purified according to previously published procedures, and their concentrations estimated as in References 9 and 14, respectively.

Washing of Thylakoid Membranes. The chloroplast pellet was finally suspended in a hypotonic medium of the desired composition. The thylakoid suspension was diluted to have a Chl concentration of 0.2 mg/ml. Aliquots of 1 ml were centrifuged (12,000g, 4 min). The supernatant was taken as the soluble phase. The chloroplast pellet was resuspended in 1 ml the same medium and was centrifuged again. The process was repeated three to five times. Chl concentration was determined (27) at each step by extracting an aliquot of 0.1 ml with 0.9 ml 90% acetone.

Reconstitution Experiments. The reconstitution reaction was performed in a medium (0.2 ml) containing 1 mm Tricine-NaOH (pH 8), 1 mm dithioerythritol, 0.2 m sucrose, 5 mm ferredoxin-NADP reductase, and chloroplasts containing 0.1 to 0.2 mg Chl/ml, unless otherwise stated. Cations were tested at the concentration indicated in the text. After 10 min at 15°C, the samples were centrifuged (12,000g, 5 min) and the membranes resuspended by adding an ice-cold solution of 25 mm Tricine-NaOH (pH 8), 250 mm sucrose, and 5 mm MgCl2.

Enzyme Assays. NADP photoreduction was measured as in Reference 11 except that the system ascorbate-dichlorophenol indophenol was employed as electron donor instead of water. NADPH-dependent diaphorase activity was assayed according to
Reference 13.

Chemicals. NADP(H), Mes, Tricine, dichlorophenol indophenol, dihydroxyethytritol, glucose-6-P, and glucose-6-P dehydrogenase were from Sigma Co. All other reagents were of analytical grade.

RESULTS

Solubilization of Ferredoxin-NADP Reductase from Thylakoid Membranes. Table I shows the effect of successive washes with different media on the release of ferredoxin-NADP reductase into the medium. Even in the presence of EDTA, widely used for the extraction of the enzyme in several purification procedures (1, 25, 28), almost 60% diaphorase activity was recovered in the thylakoid fraction. The amount of reductase bound to the membranes decreased slowly with the washes, but even after three washes a significant amount of reductase, 18 to 47% depending upon the washing medium, was membrane-bound (Table I). Maximal solubilization was attained with either 0.75 or 1 mM EDTA. Higher concentrations of reagent released less enzyme (data not shown).

The time course of solubilization of the reductase from thylakoid membranes is shown in Figure 1. A rapid loss of diaphorase activity from the particulate fraction was observed within the first 2 min, followed by a slower release. Intact chloroplasts were also ruptured in a medium of low ionic strength similar to that used by

Table I. Solubilization of Ferredoxin-NADP Oxidoreductase from Thylakoid Membranes

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration</th>
<th>NADP Photoreduction</th>
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<tbody>
<tr>
<td></td>
<td>mm</td>
<td>µmol NADPH·h⁻¹·mg⁻¹ Chl</td>
</tr>
<tr>
<td>None</td>
<td>11.0</td>
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</tr>
<tr>
<td>MgCl₂</td>
<td>5</td>
<td>42.0</td>
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<td>MnCl₂</td>
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<tr>
<td>CaCl₂</td>
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<td>29.0</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>5</td>
<td>38.0</td>
</tr>
<tr>
<td>Mg acetate</td>
<td>5</td>
<td>43.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>20</td>
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</tr>
<tr>
<td>Na₂SO₄</td>
<td>20</td>
<td>38.7</td>
</tr>
<tr>
<td>Tricine-NaOH</td>
<td>20</td>
<td>36.0</td>
</tr>
<tr>
<td>KCl</td>
<td>20</td>
<td>35.0</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>20</td>
<td>34.0</td>
</tr>
</tbody>
</table>

FIG. 1. Time course of solubilization of ferredoxin-NADP reductase from thylakoid membranes. Intact chloroplasts were suspended in the indicated hypotonic media. At the corresponding times, aliquots were taken and mixed with a concentrated solution of MgCl₂-sucrose-Tricine giving a final concentration of 20 mM Tricine-NaOH (pH 8), 0.2 M sucrose, 5 mM MgCl₂. The samples were centrifuged (12,000g, 4 min) and the particulate fraction, once resuspended, was assayed for diaphorase activity. Closed symbols, chloroplasts broken in 0.75 mM EDTA (pH 7); open symbols, chloroplasts broken in 1 mM Tricine-NaOH (pH 8), 5 mM dithioerythritol, 5 mM CaCl₂.

FIG. 2. Scatchard plot of the binding isotherm of ferredoxin-NADP reductase to thylakoid membranes. Intact chloroplasts were suspended in 0.75 mM EDTA (pH 7), and washed twice with the same medium. r, bound enzyme (nmol reductase/µmol Chl); c, free reductase at equilibrium (µM) Details concerning the calculation of c and r are given in the text. Open symbols, 0.2 mg Chl/ml; closed symbols, 0.084 mg Chl/ml.

Reconstitution experiments were carried out as described in the text with twice washed thylakoid membranes and the additions stated.

Rebinding of Ferredoxin-NADP Reductase to Depleted Spinach Chloroplasts

Reconstitution with soluble flavoprotein markedly increased NADP photoreduction in the particulate fraction. Rebinding was maximum only when cations were present in the medium. How-
were described under "Materials and Methods." A, closed symbols, MgCl₂; open symbols, NaCl. B, closed symbols, La(NO₃)₃.

Table III. Stimulation of Diaphorase Activity by Depleted Thylakoid Membranes

Chloroplasts were processed as in Figure 1 and recombined with either their soluble phase or purified enzyme in a final volume of 0.2 ml. Chl concentration was 0.25 mg/ml. MgCl₂ (5 mM) was added to the reconstitution medium. Aliquots were assayed for diaphorase activity and after centrifugation the pellet was resuspended and assayed for NADP photoreduction.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Diaphorase Activity</th>
<th>NADP Photoreduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔA/min</td>
<td>μmol NADPH·h⁻¹·mg⁻¹·Chl</td>
</tr>
<tr>
<td>1. CaCl₂-chloroplasts</td>
<td>14.0</td>
<td>30.0</td>
</tr>
<tr>
<td>2. Soluble phase of No. 1</td>
<td>8.0</td>
<td>43.5</td>
</tr>
<tr>
<td>3. No. 1 ± No. 2</td>
<td>30.0</td>
<td>31.0</td>
</tr>
<tr>
<td>4. EDTA-chloroplasts</td>
<td>8.5</td>
<td>24.0</td>
</tr>
<tr>
<td>5. Soluble phase of No. 4</td>
<td>12.5</td>
<td>79.0</td>
</tr>
<tr>
<td>6. No. 4 ± No. 5</td>
<td>28.0</td>
<td>60.0</td>
</tr>
<tr>
<td>7. Soluble enzyme (5 μm)</td>
<td>96.0</td>
<td>79.0</td>
</tr>
<tr>
<td>8. No. 1 ± No. 7</td>
<td>158.0</td>
<td>79.0</td>
</tr>
<tr>
<td>9. No. 4 ± No. 7</td>
<td>144.0</td>
<td>60.0</td>
</tr>
</tbody>
</table>

ever, little or no specificity was observed with cations of the same valency (Table II), suggesting that the effect of ions may be attributed mainly to a general electrostatic mechanism rather than to a specific chemical reaction. According to this assumption, cations of higher valency should be more effective at promoting the rebinding than those of lower valency. This is in fact the case as is shown in Figure 3, A and B, for trivalent, bi-, and monovalent cations. No perceptible influence of anions was observed (Table II).

When reconstitution was carried out with membranes depleted by two other alternative methods: a 30-min treatment with 0.75 mM EDTA or with 5 μM CaCl₂ (26) similar results were obtained (data not shown).

To eliminate the possible contribution of co-precipitation in our results the following experiment was carried out: two parallel samples were run in complete reconstitution medium with 5 mM MgCl₂. In the first experiment, the supernatant was discarded and the pellet resuspended in reductase-free medium, while in the second experiment both fractions were recombined (final concentration of soluble enzyme was approximately 5 μm). Chl concentration was adjusted to the same value and aliquots (50 μl) were assayed for NADP reduction. If stimulation was due to soluble reductase, then the first sample should have 5 to 10 times less activity than the second one. The activities of the washed thylakoids ranged from 86 to 98% of those of the nonwashed membranes (not shown). These results clearly indicate that the membrane-bound enzyme was responsible for the enhanced activity and that the contribution of the soluble flavoprotein was negligible.

Allotopic Properties of Ferredoxin-NADP Reductase. In 1975, Schneemann and Kroghmann (23) suggested that the membrane-bound reductase should be in a different conformational state when compared with the soluble form of the enzyme. In support of this suggestion, they reported stimulation of diaphorase activity of purified reductase by depleted membranes, presumably due to the interaction between the flavoprotein and the membrane. Evidence on the allotopic properties of the enzyme were obtained from kinetic (11) and chemical modification experiments (10). It seemed interesting, therefore, to determine whether such activation occurs in our conditions for rebinding.

Table III shows that there is a good correlation between activation of diaphorase activity and rebinding, measured in the pellet as NADP photoreduction. Furthermore, the diaphorase activity of reconstituted systems was higher than the sum of the activities of soluble phases and washed thylakoids. The stimulation observed was not so high as those reported (23) presumably due to the fact that only a small percentage (20–30%) of soluble ferredoxin-NADP reductase interacts with the membranes.

The possibility of a structural interaction between reductase and the complex CF₁-CF₂ was initially suggested by Berzborn (5) who observed that direct agglutination of thylakoid membranes by monospecific antireductase antisera was only attained when the membranes were depleted of CF₁. However no effect of CF₁ on reductase binding to thylakoids was observed by us when CF₁ was added before, during, or after the incubation of the flavoprotein with the membranes.

**DISCUSSION**

The results presented in this paper on the solubilization of the ferredoxin-NADP reductase in media of low ionic strength and the nonspecific effect of cations on rebinding suggest that the process may be regulated by a general electrostatic mechanism.

An explanation for the solubilization and rebinding patterns comes from application of the diffuse layer theory of Gouy-Chapman, as described for thylakoids by Barber et al. (2, 3) and formalized by Scullerby et al. (24). This model has been applied previously to explain Chl fluorescence and thylakoids stacking (4), and CF₃–CF₂ interaction (26).

We argue that attachment of reductase to membrane strictly depends upon neutralization of negative charges on the membrane surface and probably also on the protein surface. This neutralization may be the result of a combination of cation screening and cation specific binding. Solubilization of the enzyme may result from a lowering of the positive space charge density at the membrane surface (by chelation or migration of the counterions to the bulk solution). With a concomitant increase of the coulombic repulsion between the fixed protein and its negatively charged binding site on the membrane.

Böhme (8) estimated the stoichiometry of bound reductase per Chl by immunological techniques. A similar value of 3 to 4 nmol reductase/μmol Chl is reported here from rebinding experiments. The saturation pattern observed with respect to soluble protein concentration and the existence of a definite stoichiometry suggest that there should be a rather specific binding of the flavoprotein. This aspect is particularly relevant in view of the light-driven transition of the membrane-bound enzyme described by us (10–12), because a detailed knowledge of the interaction between the reductase and its binding site may contribute to elucidate the mechanisms by which the light-induced electrochemical gradient may drive the conformational movements of the bound enzyme.

The lipidic composition of the thylakoid membrane shows a

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2 Abbreviation: CF₁, coupling factor.
striking high proportion of non-charge galactosyl diglycerides (19). It seems unlikely, therefore, that the reductase molecules should interact directly with the lipid matrix by electrical phenomena. Then there are two main possibilities, either the reductase is to some extent buried into the lipid bilayer, anchored in an unknown way which should involve coulombic forces, or there is a protein-protein interaction with a membrane component. Further investigations should be necessary to discriminate between these two possible mechanisms.

LITERATURE CITED

2. Barber, J 1980 An explanation for the relationship between salt-induced thylakoid stacking and the chlorophyll fluorescence changes associated with changes in spillover of energy from photosystem II to photosystem I. FEBS Lett 118: 1–10