Purification and Properties of the Photosystem I Reaction Center from Chloroplasts

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SUMMARY

1. A reaction center from chloroplasts was purified by means of detergent treatment, differential centrifugation, column chromatography, and sucrose gradient.

2. The reaction center is active in NADP photoreduction by ascorbate. Ferredoxin, ferredoxin-NADP-reductase, and plastocyanin were required for the reaction.

3. The preparation contains five classes of polypeptide chains with apparent molecular weights of 70,000, 25,000, 20,000, 18,000, and 16,000 as determined by gel electrophoresis in sodium dodecyl sulfate.

4. Treatment with 0.5% sodium dodecyl sulfate abolished the NADP photoreduction activity and released the low molecular weight subunits, which were removed by sucrose gradient centrifugation from the high molecular weight ones. The P~00 signal is associated with the 70,000 molecular weight polypeptide.

5. Antibody, prepared against the active reaction center, inhibited NADP photoreduction catalyzed by the purified reaction center as well as by isolated chloroplasts. The antibody interacted on immunodiffusion plates with any subchloroplast preparation capable of NADP photoreduction. It also interacted with the purified 70,000 molecular weight polypeptide.

6. It is concluded that both the primary oxidation and the primary reduction in Photosystem I are associated with the 70,000 molecular weight polypeptide.

Two photosystems of the photosynthetic electron transport chain from chloroplasts were detected and separated (1, 2).

It was established that P~00 is the photochemical reaction center of Photosystem I, and it was suggested that the P~00 signal is the expression of oxidized chlorophyll a, namely the primary electron donor of the reaction center (3). Many components have been proposed for the primary electron acceptor of Photosystem I. Subsequently the resemblance between the P~00 signal and the difference spectrum of oxidized minus reduced ferredoxin was pointed out by Ke (14), and it was suggested that the P~00 signal might be an expression of the oxidation or reduction of bound ferredoxin. Later it was suggested by Siegel et al. (15) that the ferredoxin-reducing substance might serve at a secondary electron acceptor which mediates electron transport between bound and soluble ferredoxins.

Reaction center particles from photosynthetic bacteria have been isolated and purified extensively (16-23). The preparations consist of three polypeptides with molecular weights of 27,000, 22,000, and 19,000, non-heme iron, and 4 molecules of bacteriochlorophyll per reaction center (18, 19). They are photochemically active in the oxidation-reduction of P~00 and in photooxidation of reduced cytochrome c (22).

Treatment with digitonin or Triton X-100 and differential or sucrose gradient centrifugations yielded purified Photosystem I particles from chloroplasts (24-26). Chlorophyll a to b or P~00 to chlorophyll ratios were used as criteria for purification (1, 2). Recently, using digitonin treatment and sucrose gradient centrifugation, Wessels (27, 28) separated two photosystems which were both low in chlorophyll b. In these preparations the protein profiles were not characterized. The polypeptide composition of photochemically active Photosystems I and II-enriched particles was analyzed recently in several laboratories (29-35). Multiple bands were observed on the sodium dodecyl sulfate gels, and the participation of certain polypeptides in the photosystems was deduced from their relative intensity on the gels. Takamiya (36) described purification procedures yielding chlorophyll-protein complexes with molecular weight of about 70,000 from several sources. Thornber (37) purified a chlorophyll a-protein complex with molecular weight of about 150,000 from blue-green algae which contained one P~00/80 chlorophyll a molecules. However, the purified protein-chlorophyll complex was not active in NADP photoreduction.

It is the purpose of this communication to describe a preparation of reaction centers from chloroplasts having NADP photoreduction activity and of a single polypeptide having P~00 signal.

EXPERIMENTAL PROCEDURE

Materials—Swiss chard leaves were purchased from a local market. DEAE-cellulose DE11 was obtained from BDH Chemicals and was washed and equilibrated as previously described (38). Digitonin, NADP, and Tricine were obtained from Sigma.

1 The abbreviations used are: Tricine, N-tris(hydroxymethyl)methyglycine; CF~0, chloroplast coupling factor I.
Sodium dodecyl sulfate, acrylamide, and methylenebisacrylamide were purchased from Bio-Rad. Freund's Bacto-adjuvant (complete form) was obtained from Difco Laboratories.

Preparations—Chloroplasts were prepared as described previously (39). Spinach plastocyanin was prepared according to Anderson and McCarty (40).

Analytical Methods—The Ouchterlony immunodiffusion reaction (41), NADP photoreduction (42), protein (43), and chlorophyll concentration (44) were assayed by published procedures. Gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Weber and Osborn (45). The gels were fixed, stained, and destained as previously described (38). Non-heme iron was determined according to Brumby and Massey (46).

Pigments were extracted with 90% acetone. To 20 ml of the acetone extract were added 4 ml of petroleum ether (b.p. 40-60°) and it was mixed on a vortex shaker. Four milliliters of water and 2 ml of 4 M NaCl were added and mixed and the upper layer, which was formed, was transferred to a test tube. Four milliliters of water were added and the test tube was shaken on a vortex. The upper layer was transferred and solid Na₂SO₄ was added to it. The volume of the pigment extract was reduced to the desired concentration under reduced pressure at 20°.

The pigments were separated by thin layer chromatography with kieselguhr according to Randerath (47). Quinones were separated by thin layer chromatography with silica gel according to Dilley (48). The separated pigments were dissolved in appropriate solvent and their absorption spectra were recorded with a Cary 14 spectrophotometer. The pigment concentrations were calculated using absorption coefficients reported by Davies (49) for carotenoids, Strain et al. (50) for chlorophylls, and Barr and Crane (51) for quinones. The amount of P₇₃₀ was measured by placing equal samples in identical cuvettes. After the base-line was recorded by a Cary 118C spectrophotometer using l-cm quartz cuvettes.

RESULTS

Preparation of Reaction Centers Active in NADP Photoreduction

Step I: Extraction with Digitonin—Chloroplasts were prepared from Swiss Chard leaves as described previously (39) and were suspended in a medium containing 0.4 M sucrose, 0.01 M Tricine (pH 8), 0.01 M NaCl, and 5 mM MgCl₂ at a chlorophyll concentration of 1 mg per ml. Digitonin was added as a 10% solution in water to give a final concentration of 1%. After incubation at 0° for 1 hour the mixture was centrifuged at 30,000 × g for 10 min and the pellet was discarded. Solid NaCl and 10% digitonin were added to the supernatant to give final concentrations of 0.1 M and 1.5%, respectively. After incubation overnight in the cold room the mixture was centrifuged at 30,000 × g for 10 min, the pellet was discarded, and the supernatant was centrifuged at 150,000 × g for 2 hours. The supernatant of the high speed centrifugation was saved for use in the preparation of cytochrome f.

TABLE I

Purification of the Photosystem I reaction center from chloroplasts

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Chlorophyll</th>
<th>Protein</th>
<th>NADP</th>
<th>Total units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplasts</td>
<td>1,600</td>
<td>72,000</td>
<td>50</td>
<td>80,000</td>
</tr>
<tr>
<td>Photosystem I</td>
<td>660</td>
<td>3,200</td>
<td>80</td>
<td>48,000</td>
</tr>
<tr>
<td>After DEAE-cellulose</td>
<td>64</td>
<td>160</td>
<td>180</td>
<td>11,500</td>
</tr>
<tr>
<td>After sucrose gradient</td>
<td>21.3</td>
<td>68</td>
<td>400</td>
<td>8,500</td>
</tr>
</tbody>
</table>

Fig. 1. Absorption spectrum of the purified reaction center. The spectrum was recorded on a Cary 118C spectrophotometer using 1-cm quartz cuvettes.
or plastocyanine. The pellet was homogenized in a medium containing 0.4 M sucrose, 0.01 M Tricine (pH 8), and 0.01 M NaCl at a chlorophyll concentration of 3 mg per ml and was kept frozen at -70°C for as long as six months.

**Step II: Triton Treatment and DEAE-cellulose Chromatography**

Photosystem I particles (30 ml) were thawed and Triton X-100 was added as a 20% solution to give a final concentration of 4%. After incubation overnight in the cold room (at 4°C) the solution was applied to a DEAE-cellulose column (2 x 25 cm) which was equilibrated with a solution containing 50 mM Tris-Cl (pH 8) and 0.2% Triton X-100. The column was washed with 100 ml of the same buffer and the reaction center was eluted with a linear NaCl gradient from 0 to 400 mM (200 ml in each chamber) in the same buffer.

**Table II**

<table>
<thead>
<tr>
<th>Chemical composition of the Photosystem I reaction center and of the active fractions after DEAE-cellulose</th>
<th>After DEAE-cellulose</th>
<th>Reaction center</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a</td>
<td>82</td>
<td>98</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>p-Carotene</td>
<td>9.2</td>
<td>12.8</td>
</tr>
<tr>
<td>Lutein</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Iron</td>
<td>1.7</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**Step III: Sucrose Gradient Centrifugation**

The most active fractions were applied (0.5 ml to each tube) on gradients of 5 to 25% sucrose in 50 mM Tris-Cl (pH 8) and 0.2% Triton and centrifuged for 15 hours in an SW 50 Spinco rotor at 35,000 rpm. Fractions of 0.5 ml were collected and the active fractions (the lower green band) were kept at 0°C in the dark for up to 3 days.

Table I summarizes the purification procedure for Photosystem I reaction centers. It can be seen that the specific activity of the purified preparation is increased about 8-fold on a chlorophyll basis and about 10-fold on a protein basis. Its spectrum, given in Fig. 1, shows an absorption maximum at 673.5 nm and limited amounts of carotenoids. The chemical composition of the reaction center is summarized in Table II. The reaction center is almost free of chlorophyll b and the ratio of chlorophyll a to chlorophyll b is higher than 40. Neoxanthin, lutein, and violaxanthin are missing, and the amount of β-carotene is larger than in chloroplasts. Quinones could not be detected in the purified reaction center. One P100 per about 100 chlorophyll molecules was found in the preparation. Some of the chlorophyll molecules were probably solubilized since the preparation gave rather high fluorescence and rapid ascorbate photooxidation without the addition of an electron acceptor. A similar value was obtained by Vernon and Shaw (52) for the purified Photosystem I particles (TSF-I) which were prepared by Triton treatment. Unlike TSF-I particles, cytochromes could not be detected in the reaction center either by spectrophotometry or by antibody against cytochrome f (53).

The reaction center migrates nearly as a single band on acrylamide gel electrophoresis in the presence of Triton X-100, and the chlorophyll accompanies the protein band. On sodium dodecyl...
sulfate gels the reaction center dissociates to five distinct bands (Fig. 2), with molecular weights of 70,000, 25,000, 20,000, 18,000, and 16,000 (Fig. 3).

Antibody against the active reaction center was obtained by mixing it (0.5 mg protein) on a Vortex mixer with 1.5 parts of complete Freund’s adjuvant and injecting the mixture into the footpads of two rabbits and into the skin at sites on each side of the back, as previously described (38). After 4 weeks the rabbits were boosted intravenously with about 0.5 mg of the purified reaction center. The rabbits were bled as described previously (38) and boosted every month. Both rabbits gave identical antibodies even after 6 months of repeated boosting. On Ouchterlony immunodiffusion plates precipitation bands without any spur were obtained with any one of the chloroplast preparations capable of NADP photoreduction (Fig. 4). No positive reaction could be detected between the purified reaction center and antibodies against CF1 and its five individual subunits (38), cytochrome f (53), plastocyanin (54), or ferredoxin-NADP-reductase (42). The antibody inhibited NADP photoreduction not only by the purified reaction center but also by isolated chloroplasts from lettuce leaves (Fig. 5). Photophosphorylation in the presence of NADP was inhibited to the same extent as was NADP photoreduction. None of the other reactions tested including photoreduction of methyl viologen were inhibited.

Treatment of the active reaction center with 0.5% sodium dodecyl sulfate for 20 min at 0°C abolished the NADP photoreduction activity and shifted the P700 peak to 697 nm. After dilution with an equal volume of water, portions of 0.5 ml were applied on sucrose gradients as in the last step in the purification of the active reaction centers. Two green bands were formed and collected separately. The upper yellowish green band contained most of the β carotene and the four low molecular weight subunits. The lower bluish-green band contained only the 70,000 molecular weight polypeptide (Fig. 6) and one P700 per 40 to 50 chlorophyll a molecules. The antibody against the active reaction centers interacted exclusively with the fraction containing the 70,000 molecular weight polypeptide.

**DISCUSSION**

A purification procedure for the Photosystem I reaction center has been described which yields a preparation consisting of five polypeptide subunits, and which is active in NADP photoreduction. The source for the reaction center was a Photosystem I particle depleted of cytochromes and prepared by digitonin treatment and differential centrifugation (55). The DEAE-cellulose column that followed the Triton treatment removed residual cytochromes and membrane fragments which were retained on the column under the conditions described (56).
The antibody we obtained against the purified reaction center interfered with the reduction of soluble ferredoxin by chloroplasts and it might be directed toward the primary electron acceptor or toward the binding site for ferredoxin. A similar antibody was obtained by Regitz et al. (57) by injecting broken chloroplasts. However, among several injected rabbits, two gave antibodies directed against ferredoxin-reducing substance and the serum also contained antibodies against CF1 and ferredoxin-NADP reductase which had to be neutralized by adding an excess of the reductase in the NADP reduction experiments (57). One of these rabbits (6 Cl.) gave antibodies similar to those described in this paper, the other (11 Cl.) gave antibodies that inhibited not only ferredoxin-dependent reactions but also anthraquinone photoreduction (57). The antibody described in this paper was specific for subchloroplast preparations having a 700 signal and did not interact with CF1 or with ferredoxin-NADP reductase.

Using a molar extinction coefficient of 64,000 at 700 nm (14), one P700 was found per two 70,000 molecular weight polypeptides. Cooperation of two subunits might be necessary for the formation of P700, or some of the P700 molecules were lost during the purification procedure and every 70,000 molecular weight polypeptide originally had one P700. We cannot conclude yet if all of the subunits are an integral part of the reaction center; however, it seems that the 70,000 molecular weight polypeptide is necessary for NADP photoreduction. The fact that after sodium dodecyl sulfate treatment the low molecular weight subunits are missing and the P700 signal is retained means that they are not required for the primary photoreaction. It might be suggested that one or some of them are required for NADP photoreduction either to mediate electron transport from P700 to ferredoxin (acting as suggested for ferredoxin-reducing substance (6,7)) or they induce a proper conformation of the large subunit suitable for transferring electrons directly to ferredoxin. The third possibility is that they are not a part of the reaction center, and sodium dodecyl sulfate treatment partially denatured the functional subunit.

After removal of the low molecular weight subunits the antibody still interacted with the fraction which contained the 70,000 molecular weight polypeptide and P700 signal. This might suggest that both the oxidized site of Photosystem I and the primary acceptor, or at least the binding site, for ferredoxin are located on the same polypeptide.

REFERENCES

Purification and properties of the photosystem I reaction center from chloroplasts.
C Bengis and N Nelson