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NEW RAPID PROCEDURE FOR ISOLATING PHOTOSYNTHETIC REACTION CENTERS USING CYTOCHROME c AFFINITY CHROMATOGRAPHY

(photosynthetic bacteria/spinach chloroplast/membrane protein purification/detergent solubilization)

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Abbreviations: PSI, Photosystem I; PSII, Photosystem II; EPR, electron paramagnetic resonance; EDTA, ethylenediamine tetraacetic acid; Tris, tris(hydroxy-methyl)aminomethane; LDAO, lauryldimethyl amineoxide; Bchl, bacteriochlorophyll; Chl, chlorophyll; Hepes, N-2-Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; SDS, sodium dodecylsulfate; kD, kilodaltons; Rps., Rhodopseudomonas.

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ABSTRACT. Horse heart cytochrome c linked to Sepharose 4B is used to purify reaction centers from *Rhodopseudomonas sphaeroides* R-26. This procedure allows for an initial recovery of 80-90% of the bacterial reaction centers present in chromatophore membranes. High purity reaction centers (A(578 nm)/A(802 nm) < 1.30) can be obtained with a 30% recovery. Reaction centers from wild-type *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* also bind to a cytochrome c column. Cytochrome c affinity chromatography can also be used to isolate Photosystem I complexes from spinach chloroplasts.
Photosynthetic reaction centers are (bacterio)chlorophyll-protein complexes that are the sites of the photo-induced oxidation-reduction reactions that initiate the electron transport associated with photosynthesis (1). Reaction center complexes have been purified from a number of photosynthetic organisms using a variety of techniques (2-7). A recent modification of earlier techniques permits isolation of bacterial reaction centers in one day (8). In a previous application of affinity chromatography Takahashi and Gross (9) reported separations based on self-association, using either a PSII chlorophyll protein (CPII) or the light-harvesting chlorophyll a/b protein immobilized on ethylenediamine-Sepharose 4B. In this work we have utilized the known affinity of horse heart cytochrome c for bacterial reaction centers (10,11) to isolate the reaction center from Rhodopseudomonas sphaeroides R-26. We find that cytochrome c affinity chromatography can also be utilized to isolate reaction center complexes from other photosynthetic organisms, including PSI from spinach chloroplasts. The procedures described are simple and allow the rapid isolation of reaction centers without the use of precipitating agents.

METHODS AND MATERIALS

Cells of the R-26 mutant of Rps. sphaeroides grown on modified Hutner's medium (12) were harvested, washed with 100 mM Na,K-phosphate, pH 7.5, and stored in 10% glycerol at -70 C until use. The wild-type organisms were grown on yeast extract medium and harvested in the same manner.

The cytochrome c column was prepared by linking 600 mg of horse heart cytochrome c (Sigma Type VI) to 10 g of CNBr-activated Sepharose 4B (Sigma) as previously described (13). All chemicals used were of enzyme grade, when available, or of reagent grade. The pH of the buffers was adjusted at room temperature in all cases.
Absorption spectra were recorded using either a Cary 118 or a Cary 14R spectrometer. The concentration of Rps. sphaeroides R-26 reaction centers was determined from the photobleachable absorption at 870 nm using a difference molar absorptivity of 93,000 M\(^{-1}\) cm\(^{-1}\) at 870 nm (4). Side illumination of the sample cuvette by a quartz-iodine lamp with an appropriate set of filters was used to induce photobleaching, and a saturating light intensity was used in the assay. The P700 concentration was determined both by the photobleachable absorption at 700 nm and from a chemically oxidized-minus-reduced difference spectrum using a difference molar absorptivity of 64,000 M\(^{-1}\) cm\(^{-1}\) at 700 nm (14). Chl concentrations were determined by the method of Arnon (15). EPR spectra were recorded on a Varian E-9 spectrometer equipped with an Air Products Heli-Tran low temperature system.

Electrophoresis was performed in polyacrylamide gels containing 13% w/v total acrylamide with 2.7% N,N'-methylene-bis-acrylamide. Standard protein markers (Biorad) were boiled 90 s in 0.0625 M Tris-HCl/2% SDS/10% glycerol/5% 2-mercaptoethanol, pH 6.8. Reaction center samples were heated at 65 \(^\circ\)C for 1 min in the above buffer, except that 1% dithiothreitol replaced the 2-mercaptoethanol. After staining with Coomassie Blue and then destaining, the gels were perfused with 3% glycerol and dried for photography.

Rps. sphaeroides R-26 Reaction Centers. Cells, stored at 
\(-70^\circ\)C, were thawed and washed in 100 mM Na, K phosphate/10 mM EDTA, pH 7.5 (PE buffer) and resuspended in the same buffer to give 12 g cells/40 ml. After two passes through a French pressure cell (20,000 psi) the effluent was cleared of unbroken cells by centrifugation at 25,000 x g for 25 min. Intracytoplasmic membranes (chromatophores) were collected by centrifugation at 300,000 x g for 30 min and washed by resuspension in PE buffer and centrifugation to remove coupling factor and other peripheral membrane proteins. Chromatophores were
stored at -20 C in PE buffer containing 50% glycerol until use. To isolate reaction centers, chromatophores were washed once in 10 mM Hepes-NaOH/1 mM EDTA, pH 7.5 (HE buffer) to remove the glycerol, then washed in HE buffer plus 0.05% LDAO (Onyx Chemical) at a concentration to give A(865 nm) = 5.0 cm⁻¹ to further remove peripheral membrane proteins, and finally washed with HE buffer, pH 8.0. The last pellet was resuspended in 10 mM Hepes-NaOH, pH 8.0 to give A(865 nm) = 50 cm⁻¹, and a 30% w/v solution of LDAO was added to 1% w/v final detergent concentration. After stirring the suspension for 10 min at room temperature it was centrifuged at 50,000 x g for 25 min. 10 ml of the supernatant was applied to a cytochrome c affinity column (1.0 cm diameter x 2.0 cm) pre-equilibrated with 10 mM Hepes-NaOH/10 mM KCl/1% LDAO, pH 8.0. The column was washed with the equilibration buffer for approximately 5 column volumes; then it was washed with 10 mM Hepes-NaOH/10 mM KCl/0.05%LDAO, pH 8.0, until the eluent had A(280 nm) less than 0.01 cm⁻¹ (approximately 5 more column volumes). The reaction centers were eluted by raising the KCl concentration to 100 mM. The peak reaction center fractions obtained at this point have a ratio A(280 nm)/A(802 nm) = 1.5. For further purification, the reaction centers with A(280 nm)/A(802 nm) < 1.70 (approximately half the eluted reaction centers) were dialyzed against 10 mM Hepes-NaOH/0.05% LDAO, pH 8.0 to reduce the ionic strength of the solution and then reloaded onto a fresh cytochrome c column preequilibrated with 10 mM Hepes-NaOH/0.05% LDAO, pH 8.0. No protein was observed to elute from the column, and the reaction centers were immediately eluted from the column by raising the KCl concentration to 100 mM. All steps after the addition of 1% LDAO were carried out under a green safe light at 0-4 C unless otherwise noted. The same procedures were used to solubilize reaction centers from wild type organisms and bind the reaction centers to a cytochrome c column.
Spinach Chloroplast PSI Complexes. Market spinach was ground in a Waring blender for 10 s in 10 mM Hepes-NaOH/2 mM EDTA, pH 7.8, filtered through cheesecloth, and broken chloroplasts were isolated by centrifuging the sample for 10 min at 15,000 x g. The pellets were washed twice with 10 mM Hepes-NaOH, pH 7.5 and then resuspended in the wash buffer to a Chl concentration of 1 mg/ml. A 30% w/v solution of LDAO was added to 1% final detergent concentration. The sample was stirred at 0°C for 30 min and centrifuged at 50,000 x g for 30 min. 10 ml of the supernatant was applied to a cytochrome c column (1.0 cm diameter x 20 cm) pre-equilibrated with 10 mM Hepes-NaOH/1% LDAO, pH 7.5. The column was washed with 2 column volumes of the equilibration buffer. At this point the eluent had A(675 nm) < 0.4 cm⁻¹. Then the detergent concentration was decreased to 0.1% and, thereafter, the PSI reaction centers were eluted by raising the KCl concentration to 200 mM.

RESULTS

Rps. sphaeroides R-26 Reaction Centers. Pure reaction centers are most easily obtained when the chromatophores are washed free of as many proteins as possible before solubilizing the membranes and loading the detergent extract onto a cytochrome c column. Washing chromatophores with 0.05% LDAO removes a number of non-reaction center proteins; when this step was omitted we were unable to obtain pure reaction centers even after more than one passage of the sample through the cytochrome c column or use of a DEAE cellulose column after elution from the cytochrome c column. A SDS-polyacrylamide gel of the purified reaction centers is shown in Fig. 1. The three bands of apparent molecular weights 26, 29, and 31 kD correspond to the three reaction center polypeptides previously reported (16). Two very faint bands can be seen with apparent molecular weights of 49 kD and 44 kD. The 49 kD band is an aggregate of the two smallest reaction center peptides,
most likely formed during heating (17). The slight impurity with an apparent molecular weight of 44 kD can be seen as one of the predominant components of the chromatophore membrane (lane 1, Fig. 1).

Fig. 2 shows the near infrared absorption spectrum of the reaction centers eluted from the cytochrome c column, measured either in the dark or during exposure to strong light. As has been reported previously, the absorption band at 867 nm due to P870 is largely bleached upon exposure of the sample to strong light (4). The positions and relative intensities of the absorption bands both in the presence and absence of strong light are virtually the same for our preparation as for previously reported preparations (4), indicating that the reaction centers are free of light harvesting Bchl proteins or other chromophores not associated with the reaction center. The ratio \( A(280 \text{ nm})/A(802 \text{ nm}) \) is a good measure of the absolute purity of the reaction centers. We obtain a value for this ratio of 1.25 for the peak fractions obtained after two passes through a cytochrome c column. A value of 1.20 was previously reported for "pure" reaction center preparations (5).

EPR spectra were recorded for reaction centers which were frozen in the dark and then illuminated at 6 K (spectra not shown). Illumination at 6 K reversibly generated a \( g = 2.0026 \) EPR signal with a peak-to-peak linewidth of 9.8 G due to P870\(^+\). This EPR signal decayed when the light was turned off with a 1/e time of 19 ms.

The yield of reaction centers by this procedure is very high. Typically, 80-90% of the photobleachable absorbance at 870 nm present in the initial chromatophore suspension is recovered in the reaction center fractions from the first cytochrome c column. Table 1 shows the yield of reaction centers at several steps during the isolation. The loss of reaction centers which occurs when the chromatophore membranes are washed with 0.05% LDAO can
be avoided by eliminating this wash step, but then the isolated reaction centers will be only partially purified. Only half of the reaction centers eluted from the first cytochrome c column are loaded onto the second cytochrome c column. This is necessary if pure reaction centers are to be eluted from the second cytochrome c column. The capacity of the cytochrome c column was found to be greater than 0.1 mole of reaction centers/mole cytochrome c linked to the gel.

**Spinach Chloroplast PSI Complexes.** The binding constant of cytochrome c to the PSI complex is considerably smaller than that of cytochrome c to the bacterial reaction centers. Consequently, the PSI complexes begin to elute from a cytochrome c column within four column volumes, even with low ionic strength conditions. The procedure described in this work allows for the isolation of a PSI complex that is virtually free of Chl b, with approximately 60 Chl/P700 and in >90% yield. Fig. 3 shows a typical elution profile for solubilized thylakoid membranes passing through a cytochrome c column. The bulk of the Chl-containing proteins elute within one column volume, and no P700 was detected in these early fractions. Adding KCl to the elution buffer causes PSI to elute in a sharp band. It is possible to obtain samples of PSI complexes with lower Chl/P700 values by washing the material bound to the cytochrome c column for a longer period of time, but the yield is decreased. PSI complex preparations with 18-25 Chl/P700 are obtained with <10% yield after prolonged washing.

The visible absorption spectrum of ascorbate-reduced PSI complexes and the photoinduced absorption change at 697 nm are shown in Fig. 4. The absorption spectrum and photoinduced absorption changes are similar for our PSI preparation and those reported previously (6-7).
EPR spectra were recorded for the PSI preparation frozen in the dark and illuminated at 6 K (spectra not shown). No EPR signals were observed in the dark at $g = 2.00$. Illumination at 6 K irreversibly generated EPR signals at $g = 2.0025$ due to $P700^+$ and at $g = 1.86, 1.94$, and $2.05$ due to reduced iron-sulfur center A (18). Freezing a sample under illumination in the presence of 1 mM ascorbate caused the reduction of both iron-sulfur centers A and B.

**DISCUSSION**

The procedures outlined in this work allow the simple, rapid isolation in high yield of reaction centers from *Rps. sphaeroides* R-26 and PSI complexes from spinach chloroplasts. We have also found that reaction centers from wild-type *Rps. sphaeroides* and *Rps. capsulata* bind to a cytochrome c affinity column, but the eluted reaction centers were consistently contaminated with light harvesting proteins.

For the *Rps. sphaeroides* R-26 reaction centers isolated in this work we obtain a value of $A(280 \text{ nm})/A(802 \text{ nm}) = 1.25$. This value is comparable to that obtained by others for "pure" reaction centers (5). The rapid procedure of Kendall-Tobias and Seibert (8) yields reaction centers with a ratio $A(280 \text{ nm})/A(802 \text{ nm})$ between 1.4 and 1.5. The bacterial reaction centers isolated on a cytochrome c affinity column are competent in light-induced charge separation. The rate of charge recombination at low temperature in the *Rps. sphaeroides* R-26 reaction center sample (19 ms at 6 K) indicates that the charge recombination kinetics is not altered from that observed in chromatophores (19).

Although complexes containing PSI bind to a cytochrome c column substantially less tightly than do bacterial reaction centers, the strength of the interaction is sufficient to obtain a PSI sample with
approximately 60 Chl/P700 and in high yield. This PSI preparation is virtually free of Chl b (Chl a/Chl b > 10) as measured by the method of Arnon (15), indicating that there is very little contamination from the light harvesting Chla/b protein. In addition, the EPR spectrum of the PSI preparation exhibited no detectable Signal II, an EPR signal which is associated with PSII (20). The PSI complex isolated in this work contains the electron carriers that have been identified in Triton PSI particles, including iron-sulfur centers A and B. The PSI samples are also competent in light-induced charge separation at cryogenic temperatures.

We have shown that an affinity substrate, cytochrome c, can be used to purify photosynthetic reaction centers from two different classes of organisms: purple photosynthetic bacteria and chloroplasts from higher plants. The use of cytochrome c affinity chromatography may allow purification of reaction centers from a wide variety of photosynthetic organisms. A limit to the applicability of this technique may be the requirement of removing the physiological electron donor to the photosynthetic reaction center from the membrane before passing a solubilized membrane extract through the cytochrome c column.

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REFERENCES


FIGURE LEGENDS

Fig. 1. SDS-polyacrylamide gel of *Rps. sphaeroides* R-26 chromatophore membranes (lane 1), the supernatant obtained with 0.05% LDAO wash (lane 2), isolated reaction centers (lane 3), and standard protein markers (lane 4): phosphorylase B (92.5 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD).

Fig. 2. Absorption spectrum of isolated *Rps. sphaeroides* R-26 reaction centers in the dark (solid line) and during exposure to strong light (dashed line).

Fig. 3. Elution profile during passage of solubilized spinach chloroplast membranes through a cytochrome c column. The loading buffer and initial wash buffer is 10mM Hepes-NaOH/1% LDAO, pH 7.5. Detergent concentration is lowered, and salt concentration raised, as indicated by arrows. Chlorophyll concentration is monitored by the absorbance at 675nm. P700 is monitored by chemical assay.

Fig. 4. Absorption spectrum and the light-induced absorption change at 697 nm of isolated spinach chloroplast PSI complexes.
TABLE I

Yield of reaction center activity for *Rps. sphaeroides* using cytochrome c affinity chromatography. Normalization to chromatophores = 100%

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Yield of reaction Center activity&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Chromatophores</td>
<td>100</td>
</tr>
<tr>
<td>Wash with 10 mM HEPES/1 mM EDTA/0.05% LDAO</td>
<td>90</td>
</tr>
<tr>
<td>Reaction center fractions eluted from first cytochrome c column&lt;sup&gt;2&lt;/sup&gt;</td>
<td>85</td>
</tr>
<tr>
<td>Final reaction center fraction with A(280 nm)/A(802 nm) &lt; 1.30&lt;sup&gt;3&lt;/sup&gt;</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>1</sup>Average of four experiments. Activity measured as bleachable absorbance at 865 nm, measured at room temperature.

<sup>2</sup>Chromatophores were washed with EDTA and LDAO before solubilization.

<sup>3</sup>Fractions from the first cytochrome c column with A(280)/A(802)< 1.70 (approximately half of the total eluted reaction centers) were reloaded onto a second cytochrome c column.
Figure 1

92.5 kDa
66.2 kDa
45.0 kDa
31.0 kDa
21.5 kDa
14.4 kDa
Figure 3

[Graph showing elution volume (ml) on the x-axis and absorbance (A 675 nm) on the y-axis. Peaks at 0.1% LDAO and 0.2 M KCl labeled, with P700 indicated.]
Figure 4
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