Procedure for rapid isolation of photosynthetic reaction centers using cytochrome c affinity chromatography
(photosynthetic bacteria/spinach chloroplast/membrane protein purification/detergent solubilization)

G. W. BRUDEVIC*, S. T. WORLAND, AND K. SAUER†

Department of Chemistry and Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

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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_{683}/A_{605} < 1.30) can be obtained with a 30% recovery. Reaction centers from wild-type Rps. sphaeroides and Rps. capsulata also bind to a cytochrome c column. Cytochrome c affinity chromatography can also be used to isolate photosystem I complexes from spinach chloroplasts.

Photocatalytic reaction centers are (bacterio)chlorophyll (BChl)–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 by using a variety of techniques (2–7). A recent modification of earlier techniques permits isolation of bacterial reaction centers in 1 day (8). In a previous application of affinity chromatography Takahashi and Gross (9) reported separations based on self-association, using either a photosystem II (PSII) chlorophyll (Chl) protein (CPII) or the light-harvesting Chl a/b protein immobilized on ethylene diamine-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 photosystem I (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 at 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 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 by using either a Cary 118 or a Cary 14R spectrometer. The concentration of Rps. sphaeroides R-26 reaction centers was determined from the photospectrophotometric absorption at 870 nm by using a difference molar absorptivity of 93,000 M⁻¹cm⁻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 P₇₃₀ concentration was determined both by the photobleachable absorption at 700 nm and from a chemically oxidized-minus-reduced difference spectrum by using a difference molar absorptivity of 64,000 M⁻¹cm⁻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 (Allentown, PA) Heli-Tran low temperature system.

Electrophoresis was performed in polyacrylamide gels containing 13% (wt/vol) total acrylamide with 2.7% N,N’-methylenebisacrylamide. Standard protein markers (Bio-Rad) were boiled 90 s in 62.5 mM Tris HCl/2% NaDodSO₄/10% glycerol/5% 2-mercaptoethanol, pH 6.8. Reaction center samples were heated at 65°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 fused with 3% glycerol and dried for photography.

*Rps. sphaeroides R-26 Reaction Centers. Cells, stored at −70°C, were thawed and washed in 100 mM Na/K phosphate/10 mM EDTA, pH 7.5 (PE buffer) and were resuspended in the same buffer to give 12 g of cells per 40 ml. After two passes through a French pressure cell (20,000 psi; 1 psi = 6,895 Pa) the effluent was cleared of unbroken cells by centrifugation at 25,000 × g for 25 min. Intracytoplasmic membranes (chromatophores) were collected by centrifugation at 300,000 × g for 30 min and were 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 were washed in HE buffer with 0.05% lauryldimethylamine oxide (LauMe₂NO) (Onyx Chemical, Jersey City, NJ) at a concentration to give A₆₆₀ = 50 cm⁻¹ to further remove peripheral membrane proteins, and finally were washed with HE buffer (pH 8.0). The last pellet was resuspended in 10 mM Hepes/NaOH, pH 8.0, to give A₆₆₀ = 50 cm⁻¹, and a 30% (wt/vol) solution of LauMe₂NO was added to 1% (wt/vol) final detergent concentration. After stirring the suspension for 10 min at room temperature it was centrifuged at 50,000 × g for 25 min. Ten milliliters of the supernatant was applied to a cytochrome c affinity column (1.0 cm diameter

Abstracts: PSI, photosystem I; PSII, photosystem II; LauMe₂NO, lauryldimethylamine oxide; BChl, bacteriochlorophyll; Chl, chlorophyll.
* Present address: Dept. of Chemistry, Yale Univ., New Haven, CT 06511.
† To whom reprint requests should be addressed.

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equilibration buffer for until The reaction centers obtained at this point have a ratio $A_{280}/A_{902} = 1.5$. For further purification, the reaction centers with $A_{280}/A_{902} < 1.70$ (approximately half of the eluted reaction centers) were dialyzed against 10 mM Hepes/NaOH/0.05% LauMe$_2$NO, pH 8.0, to decrease the ionic strength of the solution and then were re-loaded onto a fresh cytochrome c column equilibrated with 10 mM Hepes/NaOH/0.05% LauMe$_2$NO, 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% LauMe$_2$NO 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, and filtered through cheesecloth; broken chloroplasts were isolated by centrifuging the sample for 10 min at 15,000 $\times$ g. The pellets were washed twice with 10 mM Hepes/NaOH, pH 7.5, and then were resuspended in the washing buffer to a Chl concentration of 1 mg/ml. A 30% (wt/ vol) solution of LauMe$_2$NO was added to 1% final detergent concentration. The sample was stirred at 0°C for 30 min and centrifuged at 50,000 $\times$ g for 30 min. Ten milliliters of the supernatant was applied to a cytochrome c column (1.0 cm diameter $\times$ 20 cm) equilibrated with 10 mM Hepes/NaOH/1% LauMe$_2$NO, pH 7.5. The column was washed with 2 column vol of the equilibration buffer. At this point the eluent had $A_{370} < 0.4$ 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% LauMe$_2$NO removes a number of nonreaction 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 NaDodSO$_4$/polyacrylamide gel of the purified reaction centers is shown in Fig. 1. The three bands of apparent $M_s$ 28,000, 29,000, and 31,000 correspond to the three reaction center polypeptides previously reported (16). Two very faint bands can be seen with apparent $M_s$ of 49,000 and 44,000. The $M_s$ 49,000 band is an aggregate of the two smallest reaction center peptides, most likely formed during heating (17). The slight impurity with an apparent $M_s$ of 44,000 can be seen as one of the predominant components of the chromatophore membrane (Fig. 1, lane A).

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 $P_700$ is 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 BCCh proteins or other chromophores not associated with the reaction center. The ratio $A_{280}/A_{902}$ 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 that 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 $P_700$. 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 that occurs when the chromatophore membranes are washed with 0.05% LauMe$_2$NO can be avoided by eliminating this washing 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 per mole of 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. Conse-
Fractions
Average containing passing typical approximately 60 isolation conditions. The column within consequently, the PSI complexes begin to elute from a cytochrome c column within 4 column vol, 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 per 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 1 column vol, 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 values of Chl per P700 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 per 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 = 2.005 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.

**Table 1.** Yield of reaction center activity for *Rps. sphaeroides* using cytochrome c affinity chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>RC activity, % yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatophores</td>
<td>100</td>
</tr>
<tr>
<td>Chromatophores washed with 10 mM Hepes/1 mM EDTA/0.05% LauMe2NO</td>
<td>90</td>
</tr>
<tr>
<td>RC fractions eluted from first cytochrome c column†</td>
<td>85</td>
</tr>
<tr>
<td>Final RC fraction with A436/A600 ≤ 1.30‡</td>
<td>30</td>
</tr>
</tbody>
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RC, reaction center.

* Average of four experiments. Activity measured as bleachable absorbance at 865 nm, measured at room temperature. Normalization to chromatophores = 100%.

† Chromatophores were washed with EDTA and LauMe2NO before solubilization.

‡ Fractions from the first cytochrome c column with A436/A600 ≤ 1.70 (approximately half of the total eluted reaction centers) were reloaded onto a second cytochrome c column.

**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 washing buffer is 10 mM Hepes/NaOH/1% LauMe2NO, pH 7.5. Detergent concentration is lowered and salt concentration is raised, as indicated by arrows. Chl concentration is monitored by the absorbance at 675 nm. P700 is monitored by chemical assay.

**Fig. 4.** Absorption spectrum and the light-induced absorption changes at 697 nm of isolated spinach chloroplast PSI complexes.
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 $A_{380}/A_{800} = 1.25$. 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 $A_{380}/A_{800}$ 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 per P$_{700}$ 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 Chl a/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 solubilized by using LauMe$_2$NO in this work contains the electron carriers, including iron-sulfur centers A and B, that have been identified in Triton X-100 PSI particles (6). 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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