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Reconstitution and Replacement of Bacteriochlorophyll *a* Molecules in Photosynthetic Reaction Centers

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Reaction centers (RCs) of the photosynthetic bacterium *Rhodobacter sphaeroides* R-26 were reconstituted in liposomes after release of pigments (bacteriochlorophyll *a* (BChl*a*) and bacteriopheophytin *a* (BPhe*a*)) by treatment with acetone. As shown by absorption and circular dichroism spectroscopies, the reconstituted RCs had the same arrangement of pigments as the native RC and exhibited photoactivity of the special pair. The recovery yield of RCs of up to 30% was achieved by addition of 7.8-fold excess of BChl*a* in the acetone treatment. Furthermore BChl*a* was partially replaced with Zn-BChl*a* by addition of the pigments during the acetone treatment. About 30% and 50% of the special pair and accessory pigments can be replaced with Zn-BChl*a*, respectively. From this rate, an oxidation-reduction potential of 520 mV (vs. the normal hydrogen electrode NHE) was derived by the simulation of the experimental data, which is 35 mV higher than that of the native RC (484 mV vs. NHE).

Key words: photosynthetic reaction center, reconstitution, special pair bacteriochlorophyll *a*, Zn-bacteriochlorophyll *a*.

Abbreviations: RC, photosynthetic reaction center; BChl*a,* bacteriochlorophyll *a*; BPhe*a*, bacteriopheophytin *a*; ICM, intracytoplasmic membrane; NHE, the normal hydrogen electrode; OD, optical density; ODS, octadecylsilica; OG, *n*-octyl-β-D-glucopyranoside; Q_y, the lowest π–π* transition of bacteriochlorophyll; Q_x, the second lowest π–π* transition of bacteriochlorophyll; TL, Tris-HCL buffer.

Photosynthetic reaction centers (RCs) are pigment complexes of integral membrane protein, which play key roles in the primary reactions of photosynthesis. Three-dimensional structures of several bacterial RCs have been reported, showing a symmetrical arrangement of photosynthetic pigments between the reaction center protein subunits L and M (*1–4*). Organization of the pigment molecules in the RC is fundamentally important for the efficient photo-induced electron transfer, and thus the energy transduction yield. The pigments are located on both the periplasmic and cytoplasmic sides of the membrane in the order of a bacteriochlorophyll *a* (BChl*a*) dimer called the special pair, two molecules of monomeric or accessory BChl*a*, two molecules of bacteriopheophytins *a* (BPhe*a*), and quinones (*1–4*). For elucidation of the primary reaction mechanism and/or for modification of the functions of the RC, exchange of pigment and replacement of amino acid residues around the pigment-binding sites have been actively investigated. While amino acid replacement can be achieved by conventional genetic methods, pigment exchange is much more difficult to accomplish. Exchanges of the accessory BChl*a*, BPhe*a* and quinone have been achieved by incubating the RCs with appropriate pigments in detergent solutions containing organic solvents at moderate temperatures (*5–20*). Thus, the accessory BChl*a* has been replaced with chlorophyll*a* or BChl*a* derivatives by incubating RC with the pigment derivatives in Tris-HCl buffer (TL) containing 0.06–0.1% lauryl-*N*,*N*-dimethylamine-*N*-oxide (LDAO) (*5–9*). BPhe-exchanged RCs were obtained by a similar procedure by incubating the RC with pheophytin*a* derivatives in TL buffer (*10–14*). The quinone molecules in RC were exchanged by incubating the quinone-depleted RC with orthophenanthroline and quinone derivatives in 1% LDAO (*15–20*).

Although exchange of the BChl*a* molecules in the special pairs will most critically affect the character of the RC, exchange in special pairs has not yet been accomplished. In this study, we present results of a partial replacement of BChl*a* in special pairs with Zn-BChl*a* by reconstitution of the RC protein subunits with the pigment molecules in liposomes.

MATERIALS AND METHODS

Rhodobacter (*Rb.*) *sphaeroides* R-26 was cultivated under the growth conditions previously described (*21*). Intracytoplasmic membranes (ICMs) were prepared by sonication (*22*). RCs were isolated from the ICM with the detergent LDAO (*23*). The detergent was subsequently replaced with *n*-octyl-β-D-glucopyranoside (OG) by DEAE column chromatography (*24*). BChl*a* was extracted from dry cells of *Rb. sphaeroides* R-26 with methanol and 1,4-dioxane (*25*). Zn-BChl*a* was obtained by changing the central metal ion of BChl*a* from magnesium to zinc ion (*26*). It was purified by HPLC column chromatography by

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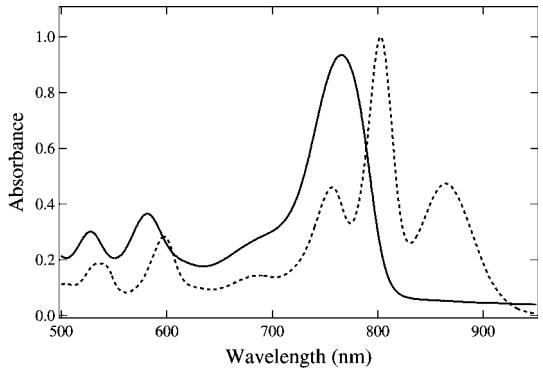

Fig. 1. **Absorption spectra of the *Rb. sphaeroides* RC before (dashed line) and after (solid line) acetone-treatment.** The untreated RC was solubilized in 15 mM phosphate buffer (pH 7.0) with 5% OG and 1 mM sodium ascorbate. The acetone-treated RC was made by mixing 300 μl of untreated RC solution and 900 μl of acetone at 0°C for 5 min.

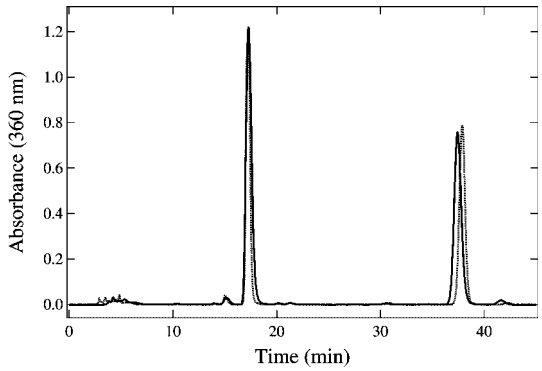

Fig. 2. **HPLC elution profiles of pigments extracted from *Rb. sphaeroides* RC.** Pigments were extracted from RCs by mixing 50 μl of RC solution (OD_{802} = 13.3) in 15 mM phosphate buffer (pH 7.0) containing 0.8% OG with 150 μl of acetone (dotted line) or 450 μl of acetone–methanol (7:2) (solid line). The detection wavelength for the HPLC was at 360 nm.

use of ODS-80Ts with acetonitrile, acetone, methanol and water (33:50:15:2, v/v) from the Zn complex of BChl*a* without the phytol side-chain, BPhe*a etc*. (*27*). In this paper, pigment exchange experiments with the purified Zn-BChl*a* are reported. Lipids were extracted from the ICM with 2-propanol, chloroform and water (1:2:1, v/v), and consist of phosphatidylcholine (27%), phosphatidylethanolamine (44%) and phosphatidylglycerol (29%).

For the pigment release, 900 μl of acetone was added to 300 μl of RC solution (OD_{802} (optical density at 802 nm) = 10) in phosphate buffer (pH 7.0) containing 5% OG and 1 mM sodium ascorbate. RCs were treated for 5 min at 0°C and the solution was lyophilized. Then 300 μl of purified water (with Millipore) was added to the dried sample and the solution was mixed gently. To this solution, 300 μl of the lipid (5–10 mg) solution in 10 mM HEPES buffer (pH 7.8) containing 0.5% (w/w) deoxycholate was added. The detergents were removed by dialysis against 10 mM HEPES buffer (pH 7.8) at 0°C for 3 d (*28*).

For the replacement of BChl*a* in the special pair and accessory position with Zn-BChl*a*, RCs were treated with acetone containing the purified Zn-BChl*a* at concentrations of 1, 5 and 7 times more than those of pigments in the RCs, then incubated in liposomes by the same procedure described above. The liposomes with reconstituted RCs were collected as pellet between centrifugation at 14,300 × *g* and at 264,000 × *g*. For characterization of the products, the reconstituted RC were resolubilized from the liposome by OG treatment (0.8%, w/v) at 0°C for 1 h and purified by a DEAE-anion exchange column chromatography (*24*).

The pigments were extracted by mixing of 450 μl of acetone–methanol (7:2, v/v) with 50 μl of DEAE-purified RC solution (OD_{802} = 1). The pigments in the native and the exchanged RCs were analyzed by HPLC column chromatography by use of ODS-80Ts with acetonitrile, acetone, methanol and water (33:50:15:2, v/v) (*27*).

To obtain difference spectra between the reduced and oxidized RCs, the exchanged RC was reduced by addition of 1 mM sodium ascorbate and oxidized by addition of 1 mM potassium ferricyanide. Absorption and circular dichroism (CD) spectra were measured with Shimadzu U-3100 and Jasco J720-W spectrometers, respectively. The photoactivity of the RCs was measured by the difference spectra between dark and light (illuminated by continuous light) absorption spectra.

The oxidation-reduction potential of the samples was determined from the absorption spectral change upon titration with reducing (sodium ascorbate) and oxidizing reagents (potassium ferricyanide) following the method of Dutton (*29*). An Ag/AgCl electrode was used for reference, and platinum wires were used as working and counter electrodes. As mediators, 20 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine and 5 mM potassium ferricyanide were used for the oxidation by the addition of potassium ferricyanide and reduction by the addition of sodium ascorbate in 15 mM phosphate buffer, respectively.

RESULTS AND DISCUSSIONS

Reconstitution of the Pigment Molecules in RCs—For the purpose of pigment exchange, we first tried to find a method to release the pigments from RCs with the least denaturing of proteins. We found that acetone was best for this purpose. Figure 1 shows the absorption spectra of the RC solution treated with three volumes of acetone in 15 mM phosphate buffer containing 5% OG as well as that of the native RC. In the near IR region, the native RC shows Q_y transitions of the special pair, accessory BChl*a* and BPhe*a* at 865, 802 and 757 nm, respectively (*23*, *30–32*). The 865 and 807 nm bands were completely bleached in the acetone-treated RC, and a new band appeared at 763 nm, near the wavelength of the Q_y transitions of BChl*a* and BPhe*a* in acetone solutions (*33*). In the visible region, the native RC shows Q_x bands of BChl*a* and BPhe*a* at 598 and 537 nm, respectively (*23*, *30–32*). The acetone-treated RC had bands in this region at 581 and 527 nm, which nearly correspond to the wavelength position of Q_x bands of BChl*a* and BPhe*a* in acetone solutions (*30–33*). From the above spectral results, it was clear that the pigments were liberated from the RC protein subunits by the acetone treatment. This is consistent with the fact that the acetone-treated RC had no

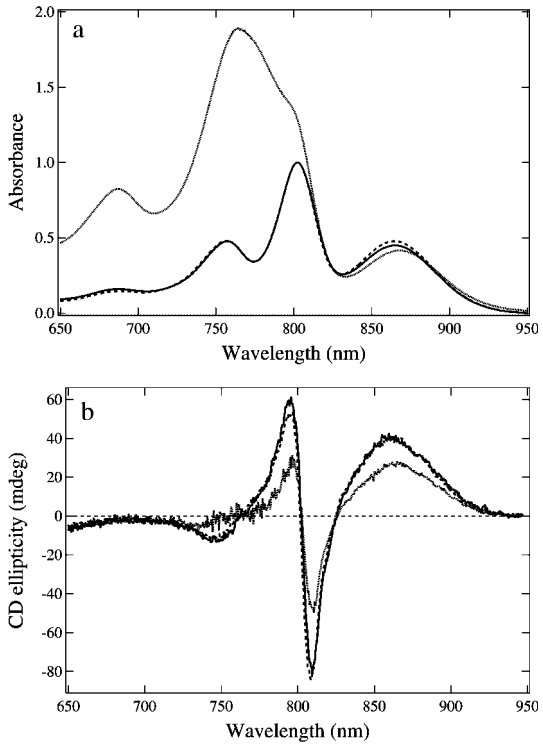

Fig. 3. **Absorption (a) and circular dichroism (b) spectra in the near IR region for the native RC (dashed line), the reconstituted RC liposome (dotted line) and the purified RC from the reconstituted RC-liposome by OG (solid line) in the presence of 1 mM sodium ascorbate.** The absorbance of the native RC, the reconstituted RC-liposome and the purified RC was adjusted to OD_{802} = 1.0 with 15 mM phosphate buffer (pH 7.0) containing 0.8% OG, to OD_{802} = 1.3 with 10 mM HEPES buffer (pH 7.8), and to OD_{802} = 1.0 with 15 mM phosphate buffer (pH 7.0) containing 0.8% OG, respectively.

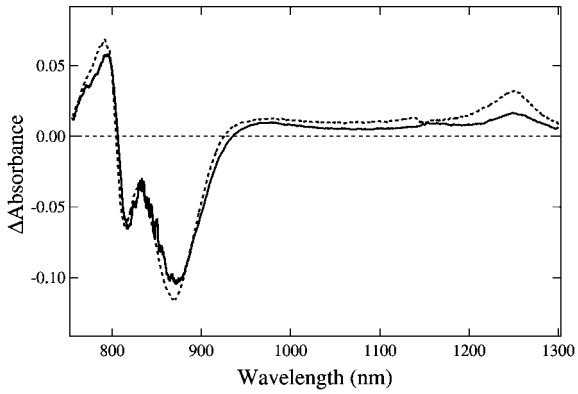

Fig. 4. **Difference absorption spectra between the reduced state by 1 mM sodium ascorbate and the photo-oxidized state in the near IR region for the native *Rb. sphaeroides* RC (dashed line) and the reconstituted RC liposome (solid line).** Spectra were measured at OD_{802} = 1.0 in 15 mM phosphate buffer (pH 7.0) containing 0.8% OG for the isolated RC and in 20 mM HEPES buffer (pH 7.8) for the reconstituted RC liposome, respectively.

photoactivity, as there was no difference in absorption between the dark and light states.

To get information about how much acetone affects the RC, the pigments extracted with acetone were compared with those extracted with the organic solvent mixture normally used for pigment extraction [acetone–methanol (7:2, v/v)]. Figure 2 shows the chromatogram of reverse-phase HPLC for the pigment extracted with acetone–methanol (7:2, v/v) from the native RCs along with that of the supernatant of the acetone-treated RC solution. The elution profiles were very similar to each other, and the two peaks were assigned to BChl*a* and BPhe*a* from absorption spectra of the fractions (data not shown). Comparison of the magnitudes of the elution peaks between the two reveals that ~90% of both BChl*a* and BPhe*a* in the RC were extracted by the acetone treatment. These results indicated that most of the BChl*a* and BPhe*a* molecules in the RC were released from their binding sites by the acetone treatment.

Intriguingly we found that the addition of liposome to the acetone-treated RC restored the absorption components of the native RC. To characterize the RC restored in the liposomes, we purified the RC and compared it spectroscopically with the native RC and RCs in liposomes. Figure 3 shows the absorption (a) and CD (b) spectra of the native RC, the acetone-treated RC reconstituted in liposomes, and the purified RC solubilized from the reconstituted liposomes with 0.8% OG. The acetone-treated RC incubated in liposomes has four peaks at 868, 800, 765 and 687 nm in the absorption spectrum and four extrema at 865(+), 810(–), 796(+) and 743(–) nm in the CD spectrum. From the peak positions of the absorption spectra and the small CD magnitude, the transitions at 765 nm and 687 nm for the liposomes with the acetone-treated RC were assigned to free BChl*a* and/or BPhe*a* and oxidized BChl*a* and/or BPhe*a*, respectively (*31*, *33–37*). On the other hand, the profiles of the absorption and CD spectra of the purified RC were very similar to those of the native RC (*31*, *35*). From the absorption and CD magnitudes of the special pair (865 nm) and the accessory BChl (802 nm) bands for the native and the purified RC, the recovery yield of RC was estimated to be 2.5 and 3.0%, respectively.

Since the recovery yield was low, we have tried to increase it. We found that addition of extra BChl*a* in the acetone treatment increased remarkably the fraction of the reconstituted RC. Thus, when a 1.5-fold excess of BChl*a* was added in the acetone, the fraction of the reconstituted RC was estimated from the absorption and CD magnitudes to be 21 and 22%, respectively, from the special pair (865 nm) and accessory BChl (802 nm) bands. With a 7.8-fold excess, it was 31 and 26%. Since the fraction of the reconstituted RC exceeds the amount of pigments remaining unextracted in the RC after the acetone treatment (Fig. 2), we conclude that RCs were reconstituted at least partially from the RC protein subunits and the released and/or added BChl*a*. The acetone treatment may release the BPhe originally present in the RC, in which case the BPhe present in the reconstituted RC would have been exchanged. This idea is also consistent with the experimental finding that the BPhe in RCs was replaced by BPhe derivatives in TL buffer with detergents, as mentioned in the introduction (*10–14*). It should

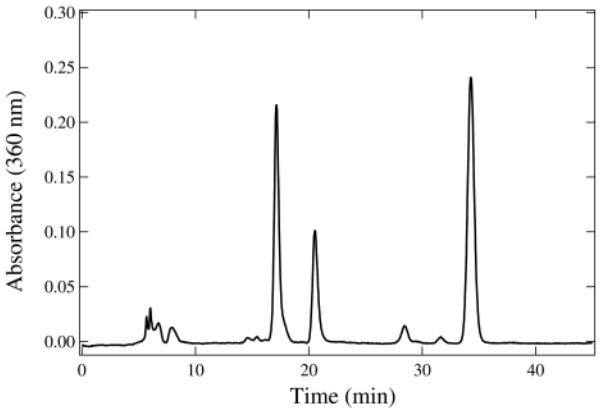

Fig. 5. **HPLC elution profiles of the extracted pigments from the exchanged RC.** The exchanged RC was adjusted to OD_{800} = 5.5, and the pigments were extracted with 50 μl of the prepared RC and 450 μl of acetone–methanol solution (7:2, v/v) at 0°C for 5 min. The elution patterns of the HPLC were detected at 360 nm.

be noted that even though the yield of 30% is still low, the reconstituted RCs could be obtained in a pure state.

In order to check the photoactivity of the recovered RC, we observed the spectral effect on the light illumination. Figure 4 shows the dark and light difference absorption spectra, which correspond to those between the reduced and oxidized states, for the reconstituted RC in the pure state and the native RC. The spectral changes of the reconstituted RC in the pure state upon illumination were very similar to those of the native RC, and were attributed to the formation of a special pair cation (1,250 nm), bleaching of the special pair (865 nm) and the blue-shift of accessory BChl*a* (800 nm) (*30*, *32*, *38*). The photoactivity of the reconstituted RC implies that the BChl*a* and BPhe*a* are reconstituted correctly in their binding site in the RC protein subunits.

Replacement of the Special Pair Pigments BChla with Zn-BChla—Since it was possible to release BChl*a* by the acetone treatment and restore it to the original positions by the incubation in liposome, we next attempted to replace the pigments with Zn-BChl*a*. Thus, the native RC was treated with acetone in the presence of Zn-BChl*a* and reconstituted in liposomes. The resultant RC was characterized by HPLC of the pigments extracted from the reconstituted RC after purification. The HPLC elution pattern is shown in Fig. 5. The results show that three main pigments are present in the purified RC, which were determined to be BChl*a*, Zn-BChl*a* and BPhe*a* from the absorption spectra of the fractions (data not shown). The magnitudes of the elution peaks revealed that the purified RC contains BChl*a* and Zn-BChl*a* in the ratio of 60:40. This suggests that Zn-BChl*a* was incorporated into the reconstituted RC in both the special pair and accessory positions at a level of 40% on average.

Figure 6 shows the absorption and CD spectra of the reduced and oxidized forms of the pigment-exchanged RC. Four bands in the absorption spectra were observed at 864, 800, 758 and 684 nm in the reduced state, corresponding to the special pair, accessory BChl*a*, BPhe*a* and oxidized BChl*a* and/or BPhe*a* (*31*, *33*–*37*). The oxidized

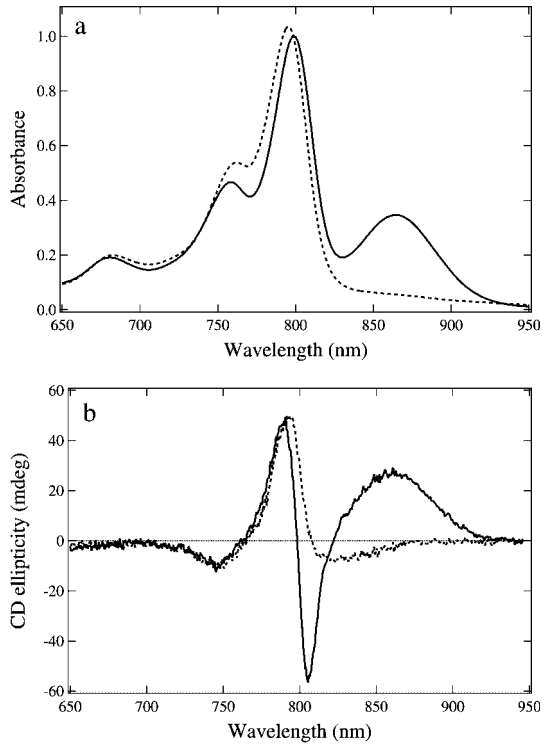

Fig. 6. **Absorption (a) and circular dichroism (b) spectra in the near IR region for the exchanged RC with Zn-BChl*a* in reduced (bold line) and oxidized (dotted line) states.** The reconstituted samples were suspended at OD_{800} = 1.0 in 15 mM phosphate buffer (pH7.0) containing 0.8% OG in the presence of 1 mM sodium ascorbate (reduced state) or 1 mM potassium ferricyanide (oxidized state).

form shows a decrease of the 864-nm band and a blue-shift of 800-nm band in comparison with the reduced form. The bleaching of the special pair band and the blue-shift of the accessory band upon oxidation imply that the exchanged RC is photoactive.

In a previous study we presented the absorption and CD spectra of the native RC from *Acidiphilium rubrum*, which has Zn-BChl*a* as the special pair and accessory BChl pigments (*39*). The CD profiles of the native RC from *A. rubrum* are very similar to those of the RC from *Rb. sphaeroides*, except for the band positions with blue-shifts of about 25 nm (for the accessory) and 7 nm (for the special pair) from those of *Rb. sphaeroides* (*39*). The CD spectra of the pigment-exchanged RC after purification are very similar to those of the native RC from *Rb. sphaeroides* with slight blue-shifts. Therefore, the CD results suggest that some fraction of the special pair and accessory BChl*a* were replaced with Zn-BChl*a* in the pigment-exchanged RC.

The exact fractions of the exchange were estimated by simulation of the absorption and CD spectra by mixing those of *Rb. sphaeroides* and *A. rubrum* in the ratio of 50:50 and 70:30. Here we have neglected the possible spectral shift due to the amino acid change. Thus, in *A. rubrum*, amino acid 168 in the L subunit is Glu, and in *Rb. sphaeroides* it is replaced by His, which is known to interact with the special pair BChl*a*. The interaction may induce some spectral shift and intensity change. This

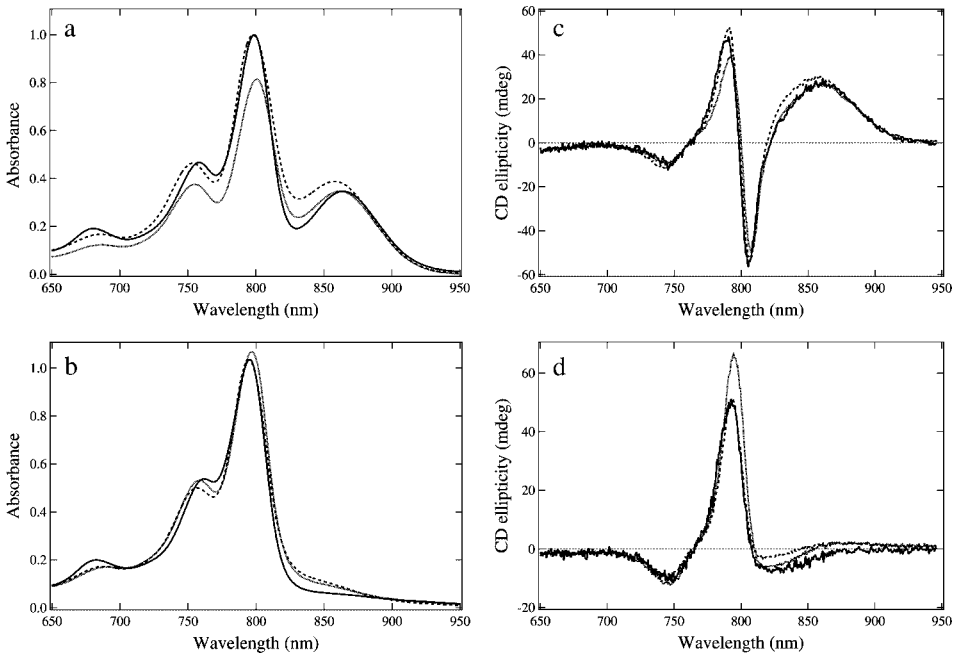

Fig. 7. **Observed and simulated absorption (a: reduced forms, b: oxidized forms) and circular dichroism (c: reduced forms, d: oxidized forms) spectra in the near IR region.** The simulated spectra are obtained by the mixing of those of *Rb.sphaeroides* and *A.rubrum* RC in ratios of 50:50 and 70:30. The observed spectra are depicted by bold lines. The simulated spectra are depicted by dashed lines for the ratio of 50:50 and dotted lines for the ratio of 70:30. The reconstituted samples were suspended at OD_{800} = 1.0 in 15 mM phosphate buffer (pH 7.0) containing 0.8% OG in the presence of 1 mM sodium ascorbate (reduced state) or 1mM potassium ferricyanide (oxidized state).

replacement may induce some red-shift for Zn-BChl*a*–exchanged *Rb*. *sphaeroides* RC, judged from the experimental results obtained when His was replaced with Asp in the RC of *Rb*. *sphaeroides* (*40*). Since we have no exact knowledge about the effect of His-to-Glu exchange in RC, we have simply neglected the effect at present as a first-order approximation. Figure 7 shows the comparison of the simulated and observed absorption and CD spectra of the reduced and oxidized forms. The peak positions and the profiles of the observed spectra were essentially reproduced at the mixing ratios of 50:50 and 70:30 of those for *Rb. sphaeroides* and *A. rubrum* for the accessory BChl and the special pair bands, respectively. The pigment composition calculated from these mixing ratios is in agreement with the pigment ratio of 60:40 for BChl*a* to Zn-BChl*a* as estimated by the HPLC analysis. All these results described above indicate the presence of Zn-BChl*a* in the reconstituted RC in the liposome. It should be mentioned that the above treatments have neglected the formation of a Zn-BChl*a* and Mg-BChl*a* heterodimer as the special pair. This discussion will have to wait until knowledge of the spectral shift and intensity change due to heterodimer formation has been obtained from model experiments.

The nature of Zn-BChl*a* in the special pair positions was examined from the oxidation-reduction behavior. Figure 8 shows the oxidation and reduction titration curves for the native RC and the exchanged RC. It is clear that the experimental points (filled triangles) for the exchanged RC do not fall on the titration curve for the native RC. In the monomeric state, Zn-BChl*a* shows an oxidation-reduction potential of 380 mV, which is 30 mV higher than that of Mg-BChl*a* (*41*). Assuming that the special pair composed of Zn-BChl*a* has a reduction potential of 515 mV, which is higher than those of Mg-BChl*a* (485 mV) (42), the experimental points in Fig. 8 fitted well with those of the simulation curve with 70% Mg-BChl*a* and 30% Zn-BChl*a* special pairs. Thus, assuming that 30% of the special pair was replaced with Zn-BChl*a*, the midpoint potential was 30 mV higher than that of the native one. Since this estimate of the fraction of Zn-BChl*a* agrees well with that from the absorption and CD spectra, the assumption that the special pair (dimer) potential is proportional to that of the monomeric states of BChl*a* and Zn-BChl*a* may be valid. The results indicate that Zn-BChl*a* special pairs do exist in the reconstructed RC and actually work as oxidation-reduction centers.

Conclusions—BChl*a* and BPhe*a* molecules in the RC were removed by acetone treatment and the removed BChl*a* and BPhe*a* were reconstituted in the RC during the reconstitution in liposomes. The photoactivity of the

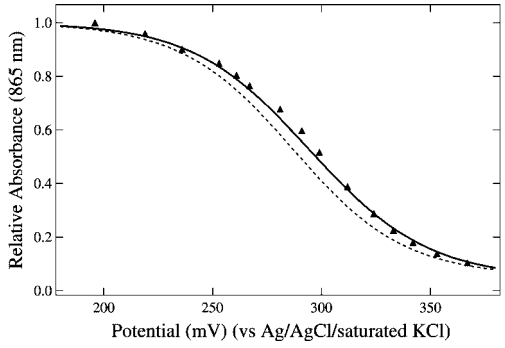

Fig. 8. **Oxidation and reduction titration curves for *Rb. shaeroides* RC (dashed line) and Zn-BChl*a* exchanged RC (filled triangles) at the concentration of OD_{800} = 1.0 for *Rb. shaeroides* RC and 0.26–0.47 for Zn-BChl*a* exchanged RC in the Q_y bands.** The solid line represents the simulated spectrum composed of the oxidation-reduction centers with oxidation-reduction potential of 287 and 313 ± 5 mV vs. Ag/AgCl in 70 and 30 ratio. The curve for *Rb shaeroides* RC has oxidation-reduction potential of 287 mV (vs. Ag/AgCl), thus 484 mV (vs. normal hydrogen electrode (NHE)), and Zn-BChl*a* was simulated with 520 ± 5 mV on the assumption of a 30 % exchange rate.

special pair was partially recovered. Partial replacement of BChl*a* in the special pair pigments with Zn-BChl*a* was also achieved. There are two types of photoactive RCs, one of which contains Zn-BChl*a* as the special pair pigments.

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