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Reaction center-B870 pigment protein complexes with bound cytochromes *c*-555 and *c*-551 from *Rhodocyclus gelatinosus*

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Pigment protein complexes which contain photochemical reaction centers and B870 light-harvesting bacteriochlorophylls were isolated from photosynthetic membranes of the purple bacterium, *Rhodocyclus gelatinosus*. The complexes consist of six kinds of polypeptide, i.e., α (molecular mass, 7.7 kDa) and β (5.8 kDa) subunits of B870 light harvesting proteins, and H (34 kDa), M (27 kDa), L (23 kDa) and cytochrome (43 kDa) subunits of the reaction-center proteins. The cytochrome subunit was quite readily dissociated from the complex by detergent treatment, and reaction-center core complexes without the cytochrome subunits were isolated from the RC-B870 complexes. Thus, the nature of the association of the cytochrome with the reaction centers in *Rc. gelatinosus* is different from that in *Rhodopseudomonas viridis* and other purple bacteria. In the reaction center-B870 complexes, four cytochrome hemes were detected per reaction center, two of which were *c*-555 (E_{m7} = 330 mV) and the other two were *c*-551 (E_{m7} = 90 mV). Both kinds of heme were oxidized in less than milliseconds by illumination with flashes.

Introduction

In purple photosynthetic bacteria, two different types of photosynthetic reaction center are known [1,2]. In one type, the reaction centers accept electrons immediately from water-soluble cytochrome c_2. *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* have reaction centers of this type [1–3]. Reaction centers of the other type have tightly bound *c*-type cytochromes which donates electrons to the photooxidized reaction centers. Reaction centers of *Rhodopseudomonas viridis*, *Rhodopseudomonas acidophila* and *Chromatium vinosum* are examples of this type [1–7]. In these species, water-soluble cytochromes c_2 were shown to be electron donors to the *c*-type cytochromes associated with the reaction centers [3,8,9]. Phylogenetic significance of the type of reaction center has been suggested recently by Matsuura and Shimada [3].

According to Woese et al. [10–12], purple photosynthetic bacteria comprise three distinct groups (α, β and γ) based on the sequence of 16 S ribosomal RNA. *Rb. sphaeroides* and *Rp. viridis* belong to group α, and reaction centers of this group have been studied extensively [1–7]. Some characteristics of the reaction centers have also revealed in *C. vinosum*, a member of group γ. On

Abbreviations: BChl, bacteriochlorophyll; RC, reaction center; SDS, sodium dodecylsulfate; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; HNQ, 2-hydroxynaphthoquinone; PMS, *N*-methylphenazonium methosulfate; TMBZ, 3,3′,5,5′-tetramethylbenzidin; Mops, 4-morpholinepropanesulfonic acid.

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the other hand, only limited information is available about the reaction centers of group β, in which *Rhodocyclus gelatinosus* and *Rhodocyclus tenuis* are included [11–14].

From the membranes of *Rc. gelatinosus*, Clayton and Clayton [15] isolated a reaction-center preparation which consists of two protein subunits. The subunits are thought to correspond to the L and M subunits of the reaction centers in α group based on their constituents and properties [7,15–17]. On the other hand, Dutton et al. [1,18–20] have shown, using the method of flash photometry at liquid nitrogen temperature, 77 K, that cytochromes are tightly linked to the reaction centers in the cells of *Rc. gelatinosus*. Clayton and Clayton [15] pointed out the possibility that the cytochromes were lost during the preparation procedure and that *Rc. gelatinosus* differs from the other species in the manner in which its *c*-type cytochromes are held in the membrane.

In order to clarify the reaction centers in the species of group β of purple bacteria further, we isolated the reaction center with the association of B870 light-harvesting pigment protein as a stoichiometric complex from *Rc. gelatinosus*. A cytochrome subunit is included in this complex and the subunit can be easily dissociated from the complex by detergent treatments.

Materials and Methods

Rhodocyclus gelatinosus IL 144 isolated by Y. Hoshino in this laboratory [21] was grown photoheterotrophically at 30 °C in glass bottles with a medium composed of 0.5% (w/v) polypepton, 0.1% yeast extract and 0.4% sodium lactate (pH 7.0).

Cells were harvested and washed with distilled water, and resuspended in 25 mM sodium phosphate buffer (pH 7.8)/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride. After disruption of cells by sonication, membrane fragments were collected by the method of a differential centrifugation as a sedimented fraction between $12\,000 \times g$ for 15 min and $144\,000 \times g$ for 1 h.

The membrane fragments were suspended in 25 mM sodium phosphate buffer (pH 7.8)/1 mM EDTA to give an absorbance at 860 nm of about 60. The suspension was cooled on ice and mixed with an equal volume of precooled mixture of 2% octyl thioglucoside (Dotite, Japan), 1 M urea and 1% Triton X-100. After immediate centrifugation for 30 min at $200\,000 \times g$, the supernatant were applied to a preparative polyacrylamide gel electrophoresis following the methods of Davis [22] with the gel buffers containing 0.1% Triton X-100 and the acrylamide concentration of 5.7%. The upper of the two major pigmented bands was extracted in the phosphate buffer and concentrated as described previously [3,23,24]. After a re-electrophoresis with the same procedure, the crude RC-B870 complexes obtained were applied on a DEAE-Sephacel column equilibrated with 10 mM sodium phosphate buffer (pH 7.3) containing 0.05% Triton X-100, eluted with the buffer containing 25 mM NaCl and purified RC-B870 complexes were obtained.

The reaction center core complexes with two polypeptides were isolated from the RC-B870 complexes by a preparative re-electrophoresis after a further treatment with 1% octyl thioglucoside/0.5 M urea/0.5% Triton X-100 in which about a half of the RC-B870 complexes were disintegrated. Reaction-center core complexes were obtained as the most mobile pigment-protein band in the electrophoresis.

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [25] and heme staining was performed by the method of Thomas et al. [26].

Absorbance changes of cytochromes started by a xenon flash (7 μs half-maximal duration, Sugawara DSX-240A) were measured with a single-beam spectrophotometer assembled in this laboratory [3]. Potentiometric titration was carried out as descirbed by Dutton [27].

Results

Fig. 1 shows the electrophoretic pattern of the Triton electrophoresis of the extracts with octyl thioglucoside and Triton X-100 from the membranes. Four pigmented bands were observed, although the most mobile one is hard to observe in the printed figure. They were identified as, from the top in Fig. 1, RC-B870 complexes (a), B800-850 complexes (b), reaction-center core complexes (c) and free pigments (d) based on the absorption spectrum of the extract of each band. The RC-B870

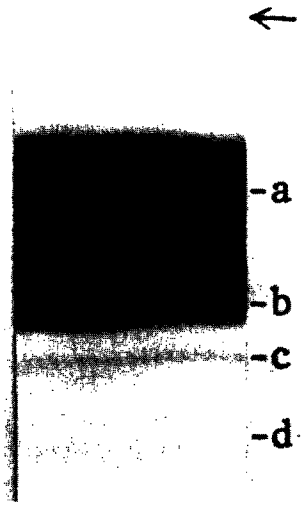

Fig. 1. Pigmented bands resolved by electrophoresis of solubilized membrane fragments of *Rc. gelatinosus*. (a) RC-B870 complexes; (b) B800-850 complexes; (c) RC core complexes; (d) free pigments. The arrow indicates the top of the running gel. Supernatant after the ultracentrifugation of membrane fragments treated with 1% octyl thioglucoside/0.5 M urea/0.5% Triton X-100 was applied on the gel containing 0.1% Triton X-100. See Materials and Methods for detail.

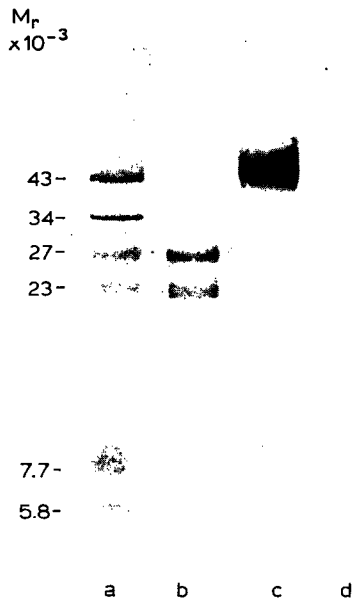

Fig. 2. Peptide compositions and heme staining patterns of RC-B870 complexes and RC core complexes resolved by SDS-electrophoresis. (a) RC-B870 complexes, Coomassie blue staining; (b) RC core complexes, coomassie blue staining; (c) RC-B870 complexes, TMBZ-H_2O_2 staining; (d) RC core complexes, TMBZ-H_2O_2 staining. RC-B870 and RC core complexes were treated with 1% SDS at 100 °C for 1 min and applied to the gel.

complexes were further purified with the second electrophoresis and a DEAE-Sephacel column chromatography and used for further experiments.

The polypeptide compositions of the RC-B870 complexes and the reaction-center core complexes are shown in Fig. 2 together with the results of heme staining of the peptides. In six major polypeptides of the RC-B870 complexes (Fig. 2a), two peptides with apparent molecular masses of 7.7 and 5.8 kDa were estimated to be α- and β-polypeptides of B870 proteins [28,29]. The other four bands are probably the components of the reaction center complexes with cytochrome subunits, although such complexes could not be isolated without the association of the B870 peptides. The two peptides, of 23 and 27 kDa, correspond to those of the reaction-center core complexes (lane b and Clayton and Clayton [15]) and were estimated to be L and M subunits of the reaction centers of other species [7,15–17], respectively. The peptide at 34 kDa possibly corresponds to the H subunit of the reaction center, as in *Rb. sphaeroides* or *Rp. viridis*, but further investigation is needed to clarify this possibility. The largest peptide, at 43 kDa, showed the heme-associated peroxidase activity (lane c) and identified as a cytochrome subunit. No such activity was observed in the peptides of the reaction-center core complexes (lane d). The relative amounts of the six major polypeptides were almost constant, independent of the preparations, indicating that these peptides form a stoichiometric complex in the membrane.

Fig. 3 shows the absorption spectrum and the reduced-minus-oxidized spectrum of RC-B870 complexes. The shape of the absorption spectrum in the infrared region is similar to that of RC-B870 complexes of other purple bacteria [3,23,24]. This means that the ratio of the reaction center to B870 is constant independent of the bacterial species and that a total of 28 bacteriochlorophylls per reaction center in the RC-B870 complexes could be estimated [3,28,29]. The difference spectrum showed the α-band maximum of *c*-type cytochrome at 553 nm. The content of the *c*-type hemes was calculated to be 3.4 per reaction center when the absorption coefficients of the hemes and

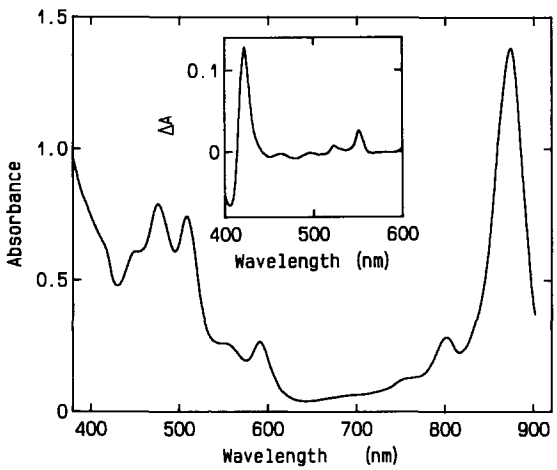

Fig. 3. Absorption spectrum and reduced-minus-oxidized spectrum of RC-B870 complexes isolated from *Rc. gelatinosus*. The complexes were dissolved in 10 mM sodium phosphate buffer (pH 7.4)/0.05% Triton X-100. The reduced-minus-oxidized spectrum was recorded after the addition of a few grains of sodium dithionite by subtracting the spectrum in the presence of potassium ferricyanide.

bacteriochlorophylls in the complexes were assumed to be 19.0 mM^{-1} [30] at 553–540 nm and 118 mM^{-1} at 870 nm [31], respectively.

The absorption spectrum of the purified reaction-center core complexes (Fig. 4) shows a typical infrared spectrum of the reaction center of purple bacteria. The cytochrome content in the preparation was less than 3% of the reaction center. This preparation showed a light-induced oxidation of the bacteriochlorophyll dimer in the reaction center identical to that of *Rb. sphaeroides* (data not shown).

Fig. 5 shows the redox titration of cytochromes in the dark in the RC-B870 complexes. Two cytochromes, with midpoint potentials of 330 mV and 90 mV, were detected. The reduced-minus-oxidized difference spectra of the two cytochromes were obtained as the differences of spectra at ambient potentials between −50 and 220 mV and 220 and 420 mV (Fig. 6). High potential cytochrome *c*-555 and low-potential cytochrome *c*-551 were identified from these spectra. These cytochromes probably correspond to cytochromes *c*-553 and *c*-548 reported by Dutton [1,20] in which peak wavelengths were measured at 77 K. The amount of cytochromes were calculated to be 1.6 *c*-555 and 2.0 *c*-551 per reaction center when the same absorption coefficient (19.0 mM^{-1}) at the peak wavelength was used with the reference at 540 nm.

Fig. 7 shows the flash-induced absorbance changes in the α-band region of the cytochromes in RC-B870 complexes (556.5–542 nm), together with the changes of the bacteriochlorophyll dimers (542 nm). These changes indicate that the *c*-type cytochromes are rapidly oxidized by the photo-

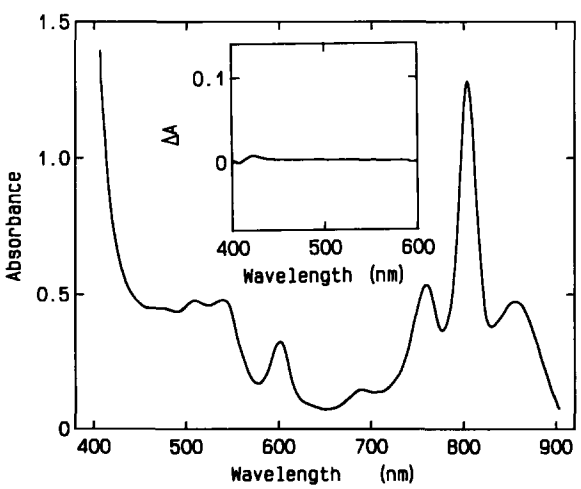

Fig. 4. Absorption spectrum and reduced-minus-oxidized spectrum of RC core complexes isolated from RC-B870 complexes of *Rc. gelatinosus*. Conditions were the same as those in Fig. 3.

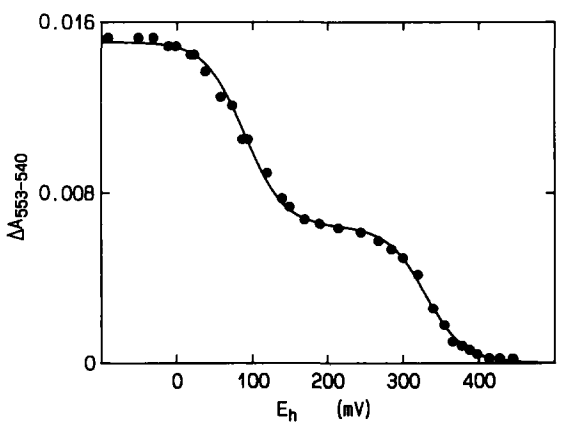

Fig. 5. Redox titration of cytochromes in isolated RC-B870 complexes of *Rc. gelatinosus* in the dark. RC-B870 complexes were dissolved anaerobically to 8.5 μM BChl in 100 mM KCl, 1 mM $MgCl_2$ and 10 mM Mops-Na (pH 7.0), containing 100 μM Fe-EDTA (1 mM) and 50 μM each of DAD, PMS, vitamin K-3 and HNQ as redox mediators.

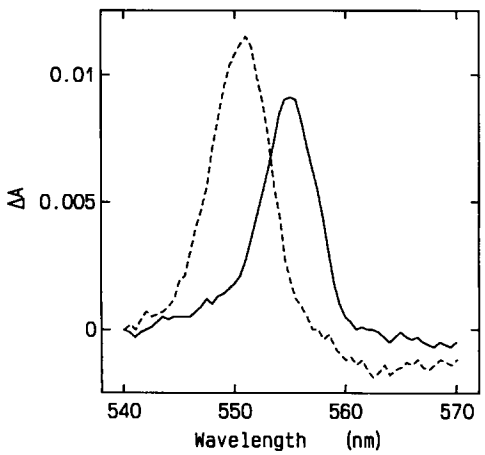

Fig. 6. Reduced-minus-oxidized spectra of cytochromes in RC-B870 complexes of *Rc. gelatinosus*. From the anaerobic redox titration shown in Fig. 5, the difference absorbances between −50 mV and 220 mV (- - - - -) and 220 mV and 420 mV (———) were recorded.

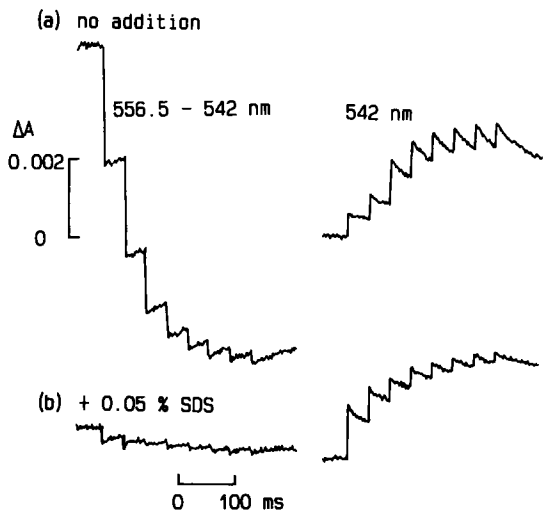

Fig. 7. Oxidations of cytochromes (556.5–542 nm, downward direction) and BChl dimers (542 nm, upward direction) in RC-B870 complexes induced by multiple flashes. RC-B870 complexes were dissolved in 100 mM KCl, 1 mM $MgCl_2$ and 10 mM Mops-Na (pH 7.0), containing 1 mM sodium ascorbate and 10 μM each of ubiquinone-6, vitamin K-1 and DAD. Eight single-turnover flashes were fired successively with the space of 40 ms. (a) No addition; (b) after the addition of 0.05% SDS and 0.1% octyl glucoside. 0.1% octyl glucoside alone did not cause any effect on the kinetic traces in (a) and 0.05% SDS alone elicited a gradual irreversible damage on the oxidation of BChl dimer in addition to the loss of the cytochrome oxidation.

oxidized bacteriochlorophylls. At the ambient redox potential of 220 mV, used for the kinetic measurements, the high-potential cytochrome *c*-555 are almost fully reduced before illumination. A single near-saturating flash elicited the oxidation of about 40% of the cytochrome. This supports the idea of the presence of two hemes of the high-potential cytochrome per reaction center. When 0.05% SDS was added to the sample, hardly any oxidation of the cytochrome was observed, although the oxidation of the bacteriochlorophyll dimer still occurred (Fig. 7b). This indicates that the cytochrome subunit can be easily dissociated with the aid of a low concentration of detergents. Octyl glucoside or Triton X-100 up to 0.1% did not show such an effect, but lauryldimethylamine oxide (LDAO) showed an effect similar to that of SDS.

E_h dependences of the flash-induced absorbance changes are shown in Fig. 8 and the spectral changes at two ambient redox potentials are shown in Fig. 9. Below E_h values around 400 mV, cytochrome *c*-555 became to be oxidized by a flash. The flash-oxidized component was changed from cytochrome *c*-555 to *c*-551, with an apparent midpoint potential around 110 mV. These depen-

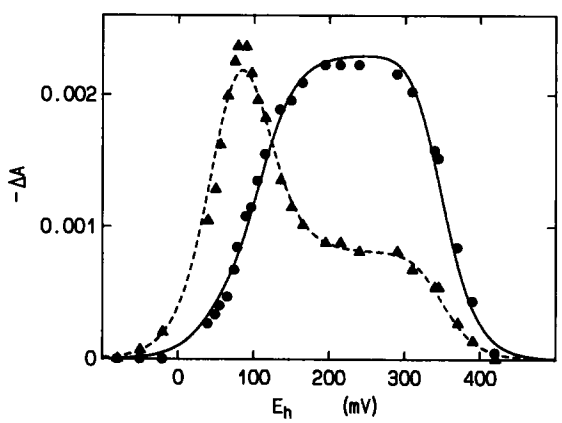

Fig. 8. Redox titration of flash-induced oxidation of cytochromes in isolated RC-B870 complexes of *Rc. gelatinosus*. RC-B870 complexes were dissolved anaerobically to 8.5 μM BChl in 100 mM KCl/1 mM $MgCl_2$/10 mM Mops-Na (pH 7.0), containing 50 μM Fe-EDTA (0.5 mM), 20 μM DAD, 5 μM PMS, 20 μM HNQ, 10 μM ubiquinone-6 and 10 μM vitamin K-1. Difference absorbance changes of the wavelength pairs, 557–542 nm (●———●) and 549–542 nm (▲- - - - -▲) were taken at 5 ms after the first flash from kinetic traces similar to those in Fig. 7.

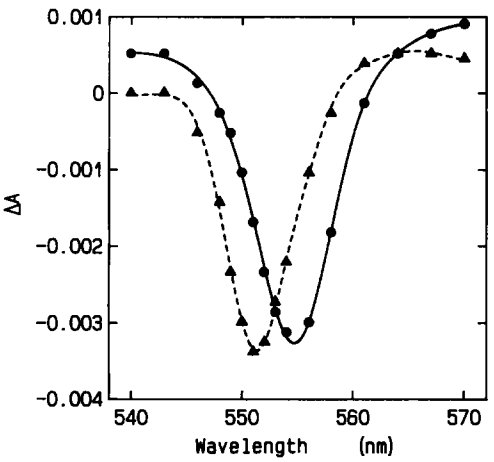

Fig. 9. Spectra of the flash-induced changes in isolated RC-B870 complexes of *Rc. gelatinosus* at ambient redox potentials of 220 mV (●———●) and 60 mV (▲- - - - -▲). Experimental conditions were the same as those in Fig. 8. Absorbance changes taken at 5 ms after the first flash were plotted against wavelength.

dences are similar to those reported in other purple bacteria which have tightly bound cytochromes *c* in the reaction-center complexes [3,5].

Discussion

We isolated RC-B870 complexes with bound cytochromes *c* from *Rc. gelatinosus*. The estimated number of hemes per reaction center, four, and the redox properties of the cytochromes are essentially the same as those of the reaction centers in *Rp. viridis* [1,4,7], *Rp. acidophila* [3] and *C. vinosum* [1,4,6], which have tightly-bound c-type cytochromes in the reaction center complexes. The staining for H_2O_2 peroxidase activity with TMBZ (Fig. 2) has revealed that the 43 kDa polypeptide corresponds to the *c*-type cytochromes. This molecular mass is also close to those of *Rp. viridis* and *C. vinosum* in which the apparent masses of the RC-bound cytochromes were estimated to be 38 and 45 kDa, respectively. On the other hand, reaction-center core complexes released from RC-B870 complexes or directly from membranes did not retain the *c*-type cytochromes in *Rc. gelatinosus* (Fig. 2) as previously reported by Clayton and Clayton [15], who isolated a reaction-center preparation from a carotenoid-less mutant using the detergent, lauryldimethylamine oxide. The association of the cytochrome subunit to the photochemical core complex is clearly more susceptible to detergents in *Rc. gelatinosus* than in *Rp. viridis, Rp. acidophila* or *C. vinosum*, which have tightly associated cytochromes in the isolated reaction centers [1,3–7].

In addition to some detergents, chaotropic agents, NaSCN or $KClO_4$, which are known to disturb hydrophobic interactions, also released the cytochrome subunit from the RC-B870 complex in *Rc. gelatinosus* (data not shown). On the other hand, an increase in ionic strength (up to 2 M NaCl) in the medium did not affect the cytochrome oxidation by flash illumination. These observations suggest that the association between the cytochrome subunit and the reaction-center core complex is not due mainly to ionic interactions, but due at least partly to hydrophobic interactions.

According to Michel et al. [7], the cytochrome subunit in *Rp. viridis* locates at the periplasmic side of the membrane without any insertions of peptide chains into the hydrophobic region of the lipid bilayer, this being based on the three-dimensional structure of the reaction-center complex. Recently, it was reported that diacylglycerol is bound to the N-terminal amino acid of the cytochrome subunit [32]. If such chains are missing in *Rc. gelatinosus*, it seems an interesting hypothesis that the presence or absence of fatty-acid chains plays a significant role in the interaction of the cytochrome subunit with the reaction-center core complex.

In purple bacteria, the role or the necessity of the cytochromes bound to the reaction centers is not yet fully understood. In *Rb. sphaeroides, Rb. capsulatus, Rp. palustris* and *Rs. rubrum*, soluble cytochromes c_2 are direct electron donors to the photooxidized bacteriochlorophyll dimers [1–3]. Cytochromes c_2 are also present in other purple non-sulfur photosynthetic bacteria, in which they function as the secondary electron donors [3,8,9]. The facilitated dissociation of the bound cytochrome from the RC-B870 complexes without significant damage of the reaction center in *Rc. gelatinosus* seems to provide a useful system to study the detailed function of the bound cytochromes.

From the comparison of 16 S rRNA sequences,

the purple non-sulfur bacteria are classified into two groups, α and β [10–12]. *Rc. gelatinosus* belongs to the β group and shows a few characteristics different from species in the α group, such as the finger-like intracytoplasmic membrane structure and a short type of cytochrome c_2 [13,14]. As for the components of reaction centers, *Rc. gelatinosus* has the bound cytochromes *c* like *Rp. viridis*, but the interaction between the reaction centers and the cytochromes is different from that in the α group species. The type of the reaction centers in terms of the tightness of association of the cytochrome subunit may be another phylogenetically significant characteristic of the β group of purple photosynthetic bacteria.

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