Supramolecular organization of the photosynthetic apparatus of *Rhodobacter sphaeroides*

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Native tubular membranes were purified from the purple non-sulfur bacterium *Rhodobacter sphaeroides*. These tubular structures contain all the membrane components of the photosynthetic apparatus, in the relative ratio of one cytochrome *bc*₁ complex to two reaction centers, and ~24 bacteriochlorophyll molecules per reaction center. Electron micrographs of negative-stained membranes diffract up to 25 Å and allow the calculation of a projection map at 20 Å. The unit cell (*a* = 198 Å, *b* = 120 Å and *γ* = 103°) contains an elongated S-shaped supercomplex presenting a pseudo-2-fold symmetry. Comparison with density maps of isolated reaction center and light-harvesting complexes allowed interpretation of the projection map. Each supercomplex is composed of light-harvesting 1 complexes that take the form of two C-shaped structures of ~112 Å in external diameter, facing each other on the open side and enclosing the two reaction centers. The remaining positive density is tentatively attributed to one cytochrome *bc*₁ complex. These features shed new light on the association of the reaction center and the light-harvesting complexes. In particular, the organization of the light-harvesting complexes in C-shaped structures ensures an efficient exchange of ubiquinone/ubiquinol between the reaction center and the cytochrome *bc*₁ complex.

Keywords: cytochrome *bc*₁ complex/LH1/photosynthetic reaction center/*Rhodobacter sphaeroides*/supercomplexes

Introduction

In bacterial photosynthesis, absorption of light initiates a cyclic electron transfer coupled to proton translocation across the cytoplasmic membrane. Three membrane protein complexes are necessary to collect and convert light energy into chemical energy: the light-harvesting complexes (LHC), the reaction center (RC) and the cytochrome (cyt) *bc*₁ complex. To ensure an efficient collection of light energy, the purple photosynthetic bacteria usually contain two types of LHC; LH1 and LH2. The amounts of LH2 are modulated by several factors, such as light intensity and oxygen tension, while LH1 are synthesized in

stoichiometric amounts with the RC (Drews and Golecki, 1995). After light absorption by the LHC, the excitation energy is transferred to the RC where a charge separation occurs between donor and acceptor molecules. The photo-oxidized primary electron donor, a dimer of bacteriochlorophyll (Bchl) molecules, is reduced by the high potential carriers of the *bc*₁ complex (Meyer and Donohue, 1995) via soluble carriers such as cyt *c*₂. To complete the cyclic electron transfer, this complex is reduced in turn by the photoreduced acceptor. The mechanisms of excitonic energy migration and photoinduced electron transfer have been studied in detail using biophysical approaches, and analyzed at the molecular level following the resolution of the atomic structure of the RC, LH2 and *bc*₁ complexes (Deisenhofer *et al.*, 1985; Allen *et al.*, 1986; McDermott *et al.*, 1995; Xia *et al.*, 1997; Zhang *et al.*, 1998). However, despite the wealth of information available on the individual proteins, their supramolecular organization in the membrane remains unclear.

A first issue is the spatial organization between LH1 complexes and RCs. Native membranes and LH1–RC complexes isolated from *Rhodopseudomonas* (*Rp.*) *viridis* (Miller, 1982; Engelhardt *et al.*, 1986; Ikeda-Yamasaki *et al.*, 1998), *Ectothiorhodospira halochloris* (Stark *et al.*, 1984; Engelhardt *et al.*, 1986), *Rp. molischianum* (Boonstra *et al.*, 1994) and *Rhodospirillum* (*Rs.*) *rubrum* (Walz and Ghosh, 1997; Stahlberg *et al.*, 1998) have been analyzed by various electron microscopic techniques. All these studies reveal a single RC inside a closed ring of LH1 complexes. Organization of LH1 complexes in a closed ring was also observed by a cryo-electron microscopy study of two-dimensional (2D) crystals of *Rs. rubrum* purified complexes (Karrasch *et al.*, 1995). The 8.5 Å projection map, obtained in this latter study, showed 16 subunits arranged in a ring-like structure (116 Å overall diameter, 68 Å inner diameter) that could only accommodate one RC. The closed LH1 structure raised the question of how the quinone/quinol transfer took place between the RC acceptor site and the *bc*₁ complex during cyclic electron transfer. Several hypotheses have been proposed, such as slight movements of the α -helices allowing diffusion of the quinone molecules through the ring (Karrasch *et al.*, 1995). It has also been suggested that the presence in the ring of additional proteins such as PufX, an essential polypeptide for photosynthetic growth of *Rhodobacter* (*Rb.*) *sphaeroides* and *Rb. capsulatus* (Farchaus *et al.*, 1990; Lilburn *et al.*, 1992), or the Ω polypeptide in the case of *Rs. rubrum* (Ghosh *et al.*, 1994; Stahlberg *et al.*, 1998), may catalyze quinone transfer by forming specific channels through the ring of LH1 complexes (McGlynn *et al.*, 1996).

A second issue is the organization of the RC and *bc*₁ complexes in the photosynthetic unit. A series of experiments performed with intact cells of *Rb. sphaeroides*

Fig. 1. (A) Electron micrograph of spheroplasts of *Rb.sphaeroides* strain RCLH10. Samples were negatively stained with 1% phosphotungstic acid. Bar = 500 nm. (B) Unidirectional shadowed tubular membrane of *Rb.sphaeroides* forma sp. *denitrificans* obtained by freeze-drying. Bar = 200 nm.

prompted the proposal that the elements of the photosynthetic electron transfer chain might be organized in supercomplexes (Joliot *et al.*, 1989), each one comprising two RCs, one *cyt c₂* and one *bc₁* complex. This proposal originated from the observation that the apparent equilibrium constant between the different reactants, measured during photooxidation of the donor chain under continuous illumination, was much lower than that deduced from their redox mid-point potentials measured at equilibrium (Joliot *et al.*, 1989; Lavergne *et al.*, 1989). The supercomplex hypothesis has been challenged by Fernández-Velasco and Crofts (1991), and an alternative model was recently proposed to explain the low value of the observed equilibrium constant (Crofts *et al.*, 1998). The basic assumption in this alternative model is a heterogeneity in the ratio of electron transfer components in individual chromatophores acting as separate compartments. However, this model fails to explain the following important observations made on intact cells of *Rb.sphaeroides*: (i) the addition of subsaturating concentrations of myxothiazol, a specific inhibitor of the *bc₁* complex, decreases the number of active *bc₁* complexes but does not affect the rate of cyclic electron transfer for the uninhibited complexes (Verméglio *et al.*, 1993; Joliot *et al.*, 1996); (ii) the complete photoinduced cyclic electron flow functions even at -20°C in a frozen medium (Joliot *et al.*, 1997); (iii) evidence, by freeze-fracture electron microscopy, of a dimeric arrangement of the photosynthetic membrane

proteins (Golecki *et al.*, 1991; Sabaty *et al.*, 1994) in the tubular membranes of *Rb.sphaeroides* cells deleted in LH2 complexes (Hunter *et al.*, 1988; Kiley *et al.*, 1988) or grown in the presence of nitrate (Sabaty *et al.*, 1994); and (iv) the very recent biochemical evidence that dimeric LH1-RC complexes can be isolated by gentle solubilization of photosynthetic membranes of *Rb.sphaeroides* (Francia *et al.*, 1998).

Here, we report on the biochemical and structural characterization of these tubular membranes. A projection map at 20 Å resolution was obtained by electron microscopy of negatively stained samples. The electron density distribution agrees with the supramolecular arrangement of the photosynthetic apparatus determined by functional approaches, in particular the dimeric association of the photosynthetic RCs (Joliot *et al.*, 1989). An important additional finding is the organization of the LH1 complexes in C-shaped structures, which sheds new light on the coupling of electron transfer between RC and *bc₁* complex by the quinone molecules.

Results

Purification and biochemical characterization of the tubular membranes

The cytoplasmic membrane of *Rb.sphaeroides* forma sp. *denitrificans* grown photosynthetically in the presence of 200 mM nitrate or under dark semi-aerobic conditions

presents a large number of tubular membranes (Sabaty *et al.*, 1994). The presence of these tubes is correlated with the decreased synthesis of LH2 complexes. Similar tubular membranes were found in various mutants of *Rb.sphaeroides* in which this complex is deleted (Hunter *et al.*, 1988; Kiley *et al.*, 1988). Figure 1A shows an electron micrograph of negatively stained spheroplasts of *Rb.sphaeroides* strain RCLH10. These tubes are between 0.5 and 2 μm long and ~ 116 nm in diameter as shown by the freeze-drying electron micrograph depicted in Figure 1B (see also Golecki *et al.*, 1991). Interestingly, these freeze-drying pictures present well-organized rows of dimeric particles, in agreement with freeze-fracture experiments (Golecki *et al.*, 1991; Sabaty *et al.*, 1994). We purified these tubes from cells of *Rb.sphaeroides* forma sp. *denitrificans*, and from mutant RCLH10 deleted in LH2 complexes, as described in the Materials and methods. The purity of the preparations was assayed by electron microscopy of negatively stained samples. The best samples contained $<10\%$ contamination in the form of cytoplasmic membrane fragments and chromatophores. The upper part of Figure 2 shows the absorption spectrum of purified tubular membranes recorded at 77 K. In the near-infrared spectral region, the main absorption band is centered at 880 nm and corresponds to the Q_y transition of the LH1 complexes. In addition, typical bands of Bchl and bacteriopheophytin (Bpheo) molecules of the RC are centered at 804 and 760 nm, respectively. The blue region is dominated by the Q_x transitions of the LH1 complexes at 590 nm and by the carotenoid bands centered at 445, 472 and 505 nm. The presence of bc_1 complex is revealed by the characteristic α -bands of $\text{cyt } c_1$, $\text{cyt } b_H$ and $\text{cyt } b_L$ centered at 548, 556 and 561 nm, respectively. From five different preparations of purified tubular membranes, a relative ratio of 0.9 ± 0.05 bc_1 complex per 2 RCs was determined. This ratio did not significantly change during the purification procedure, and is similar to that measured for chromatophores of wild-type *Rb.sphaeroides* (van der Berg *et al.*, 1979). The presence of RCs, bc_1 complexes and LH1 complexes was further confirmed by gel electrophoresis (Figure 2B). This gel presents characteristic polypeptide bands of the RC at 28, 26 and 24 kDa. The bands of the $\text{cyt } b$, $\text{cyt } c_1$, Rieske protein and subunit IV were observed at 43, 33, 20 and 14 kDa, respectively. These assignments were confirmed by Western blots (not shown). LH1 subunits were detected at 11 and 8 kDa. Part of the LH1 migrates as oligomers of high molecular weight as already observed by Hunter *et al.* (1988). We also measured the relative ratio of Bchl to RC, which we found to be 24–25 Bchl molecules per RC in agreement with previous work on membrane or isolated complexes (van Grondelle *et al.*, 1994; McGlynn *et al.*, 1996; Francia *et al.*, 1998).

Electron microscopy of the tubular membrane

The high degree of organization observed by freeze-etching (Golecki *et al.*, 1991; Sabaty *et al.*, 1994) and freeze-drying (Figure 1B) suggests that the tubular membrane purified from *Rb.sphaeroides* is a natural 2D crystal of membrane proteins that can be analyzed by negative staining and electron microscopy. Figure 3A shows low-dose electron micrographs of negatively stained tubular membranes. Optical diffraction analysis of such images

Fig. 2. (A) Absorption spectrum of purified tubular membranes of *Rb.sphaeroides* recorded at 77 K. The inset represents the dithionite-reduced minus ferricyanide-oxidized difference spectrum of the same preparation in the α -band region. (B) Polypeptide composition of purified tubular membranes isolated from *Rb.sphaeroides*. RC indicates the three polypeptide subunits H, M and L of the reaction center migrating with apparent molecular weights of 28, 26 and 24 kDa, respectively. The four subunits of the $\text{cyt } bc_1$ complex are indicated by FeS, $\text{cyt } b$, $\text{cyt } c_1$ and IV. LH1 indicates the subunits of the LH1 complexes with apparent molecular weights of 11 and 8 kDa.

clearly shows two distinct patterns, both with sharp strong spots. The Fourier transform and the filtered image of one tubular crystal are depicted in Figure 3B and C, respectively. These two diffraction patterns correspond to two layered 2D crystals diffracting independently and originating from the top and bottom parts of the tube flattened on the electron microscope grid. Only the pattern with the strongest non-overlapping spots, which corresponds to the top layer, was subsequently used for image processing. The indexation clearly indicated a monoclinic unit cell with parameters: $a = 198$ Å, $b = 120$ Å and $\gamma = 103^\circ$, and the resolution extended up to 25 Å. Eight images were selected, by optical diffraction, digitized and processed using the procedure of Crowther *et al.* (1996). Five images were kept for the final merging step. Reflections with quality index for image phases (IQ) values of 5 are visible out to 20 Å resolution (Figure 4A).

Fig. 3. (A) Low-dose electron micrographs of native tubular membranes from *Rb.sphaeroides*. Samples were negatively stained with 1% uranyl acetate. Bar = 100 nm for all the tubes. (B) Fourier transform of one tube from digitized scan. The arrow indicates the resolution at 25 Å. (C) Filtered image from masked Fourier transform of electron micrograph. Bar = 200 Å.

A projection map was calculated (Figure 4B). The unit cell (198×120 Å) contains an elongated S-shaped supercomplex composed of two C-shaped structures of external diameter 112 Å, their open sides facing each other and enclosing a large protein mass. In addition to the density localized in the center of each ring, a positive density is detected between the two C-shaped structures. This S-shaped supercomplex corresponds closely to the dimeric appearance of the rows observed in freeze-dried (Figure 1B) and freeze-fractured samples (Golecki *et al.*, 1991; Sabaty *et al.*, 1994). However, at the higher resolution achieved in this study, the S-shaped arrangement displays a clear asymmetry for each individual picture (Figure 3C).

Discussion

In this work, we purified and analyzed in detail the tubular membranes of *Rb.sphaeroides* induced by the

absence of LH2 complexes. These membranes contain all the proteins necessary for photosynthetic activity: RC, LH1 and bc_1 complexes. We found a bc_1 complex to RC ratio of 0.5, and 24–25 Bchl molecules per RC. The organization of the photosynthetic unit was analyzed by electron microscopy, taking advantage of the 2D crystal organization of the native membranes. The excellent quality of these tubular membranes enabled us to record low dose images of negative stained specimens and to resolve, for the first time, the supramolecular organization of the complete photosynthetic apparatus in a native membrane. The 20 Å projection map revealed that the membrane proteins are arranged as two connected C-shaped structures enclosing a large protein mass. Taking into account the composition and stoichiometry of the membrane complexes in the tubular membranes, the following interpretation of the projection map shown in

Fig. 4. (A) Diffraction properties of negatively stained native tubular membranes from *Rb.sphaeroides*. Plot showing the Fourier transform of five negatively stained images after processing and merging. The size of the circles indicates the IQ value. The two dotted rings are at radii corresponding to $1/40$ and $1/20 \text{ Å}^{-1}$. (B) Projection map at 20 Å resolution after processing and averaging of negatively stained native tubular flat membrane from *Rb.sphaeroides*. The unit cell ($a = 198 \text{ Å}$, $b = 120 \text{ Å}$ and $\gamma = 103^\circ$) is outlined in black. Positive density representing the protein is shown as solid lines and negative density as dotted lines.

Figure 4 is proposed. Each C-shaped structure in the projection map corresponds to LH1 complexes. This attribution agrees with the 8.5 Å projection map of the LH1 complex of *Rs.rubrum* (Karrasch *et al.*, 1995) which revealed a circular arrangement of 16 transmembrane $\alpha\beta$ subunits in a 116 Å diameter ring with a 68 Å hole in the center, similar in size to that observed in our projection map. An important observation is the C-shape of the LH1 complex in the tubular membrane, as opposed to the closed ring observed for the reconstituted LH1 complexes of *Rs.rubrum*. Given that the 16 $\alpha\beta$ subunits present in the closed ring of *Rs.rubrum* LH1 correspond to 32 molecules of Bchl, and that we measured ~ 24 Bchl molecules per RC in the tubular membrane, we deduce that the C-shaped structure should contain $\sim 12 \alpha\beta$ subunits. The superimposition of $3/4$ of the projected structure of the closed ring of LH1 complexes of *Rs.rubrum* with our projection map, shown in Figure 5, supports this stoichiometry. The protein density observed within each LH1 C-shaped structure can be attributed to one RC, in agreement with previous observations in other species (Miller, 1982; Stark *et al.*, 1984; Engelhardt *et al.*, 1986; Boonstra *et al.*, 1994; Ikeda-Yamasaki *et al.*, 1998). This protein density is well resolved, suggesting that the RC can occupy a single well-defined position with respect to the LH1. The remaining density, localized between the two C-shaped structures, can tentatively be attributed to the bc_1 complex. This accounts for both the presence of the bc_1 complex in the ratio of 1:2 RCs, and for the asymmetry of the unit cell. However, at the present stage it is difficult to localize the bc_1 complex precisely in this negative-stain projection map. Because the stain does not penetrate the membrane, the density contribution in the projection map arises solely from the cytoplasmic surface of the flattened tube. The contribution of the bc_1 complex in the projection map is thus expected to be very weak

Fig. 5. Proposed model of the photosynthetic unit of *Rb.sphaeroides* viewed from above the membrane. The projection structure of the RC of *Rb.sphaeroides* (Allen *et al.*, 1987; Ermler *et al.*, 1994) and the projection map of $3/4$ of LH1 of *Rs.rubrum* (Karrasch *et al.*, 1995) are shown in red and green, respectively.

because most of the extramembranous parts of this complex (Rieske protein and $\text{cyt } c_1$) are located in the internal membrane of the tube and do not contribute significantly to the projection map.

The supramolecular organization of the photosynthetic components observed in this electron microscopic study

agrees with the suggestion, based on diverse functional evidence, that the photosynthetic electron chain of *Rb.sphaeroides* is organized in supercomplexes that include two RCs and one bc_1 complex as membrane partners (Joliot *et al.*, 1989, 1996, 1997; Verméglio *et al.*, 1993). The observation that only one cyt c_2 can bind to two RCs even when an excess of cyt c_2 is present in the periplasmic space was an important argument in favor of a dimeric association of the RCs in the supercomplex of *Rb.sphaeroides* (Verméglio *et al.*, 1993). Similarly, analysis of flash-induced absorbance changes on intact cells of *Rs.rubrum* has shown that the anticooperative interactions between the two cyt c_2 -binding sites prevent the simultaneous binding of two cyt c_2 molecules on the same dimer of RCs (Joliot *et al.*, 1990). In the case of *Rb.sphaeroides*, this behavior can be tentatively explained by postulating that the RCs in the supercomplex are positioned so that their M-subunits, and hence the interaction site between the cyt c_2 and the RC (Tiede *et al.*, 1993; Adir *et al.*, 1996) are close together. This positioning of the RCs would also allow an efficient exchange of quinone molecules between the RC and the bc_1 complex (see below). A tentative match between our density map and a projection map derived from the coordinates of the *Rb.sphaeroides* RC determined by X-ray crystallography (Allen *et al.*, 1986; Ermler *et al.*, 1994) where the M-subunit and hence the secondary electron acceptor (Q_B) sites of both RCs are facing the open side of the two LH1 rings in the pseudo-dimeric association, is depicted in Figure 5.

It must be emphasized that the functional evidence for an organization in supercomplexes of the photosynthetic electron carriers was not dependent on the LH2:LH1 ratio (Verméglio *et al.*, 1993; Sabatý *et al.*, 1994). This suggests that the supercomplex organization observed in the tubular membrane is also present in the invaginated membrane. The presence of LH2 complexes in non-stoichiometric amounts prevents a crystalline arrangement of the photosynthetic components and leads to the formation of the chromatophores. On the other hand, in the absence of LH2 the formation of the tubular membrane is induced by the crystallization of the membrane proteins present in a stoichiometric ratios. Finally, it is noteworthy that the dimeric organization of the RCs and LH1 complexes reported here is also consistent with the recent observation that dimeric cores composed of two RCs, two PufX polypeptides and ~27 LH1 subunits can be isolated from wild-type *Rb.sphaeroides* membranes after gentle solubilization (Francia *et al.*, 1998). From the functional results obtained with *Rs.rubrum* intact cells (Joliot *et al.*, 1990), we postulate that a similar dimeric association of RCs is also present in this species, although further supporting structural and biochemical evidence is required. Finally, it is also interesting to note that PSII RCs, which are highly homologous to the purple bacteria RCs, have been suggested to present a dimeric association *in vivo* where the D1/D2 heterodimers are in close contact and surrounded by LHC (Rögnér *et al.*, 1996; Hankamer *et al.*, 1997).

Besides the structural evidence for a supercomplex organization of the photosynthetic chain of *Rb.sphaeroides*, other important information can be drawn from our electron microscopy study. One striking feature is the organization of the LH1 complexes in C-shaped structures.

This finding contrasts with previous proposals based on image processing and analyses of electron microscopy pictures recorded for both highly organized native membranes and purified LH1-RC and LH1 complexes. The picture that emerges from these various studies is that the LH1 complexes form a closed ring surrounding the RC when present. In the work of Karrasch *et al.* (1995), it should be noted that the LH1 complexes of *Rs.rubrum* were dissociated into $\alpha\beta$ dimers prior to crystallization, and therefore the closed ring structure occurring in the reconstituted system gives no direct information on the arrangement in the native membrane. Similarly, the formation of a closed ring in the isolated LH1-RC complexes of other species may be induced during solubilization, as discussed by Francia *et al.* (1998). When the dimeric arrangement is disrupted by solubilization, a closed ring of LH1 completely encircling the RC may well be more stable than a C-shaped structure. In the case of *Rp.viridis*, the circular appearance of the LH1 ring could be an artifact caused by averaging. Nevertheless, at the present stage, it is difficult to know how far the organization in C-shaped structures of LH1 observed in *Rb.sphaeroides* can be generalized to other species of purple bacteria, in particular to those containing a low bc_1 :RC ratio and (or) possessing no PufX-like polypeptide.

Whatever the final answer to this question is, organization in C-shaped structures of the LH1 complexes of *Rb.sphaeroides* in native membrane has important functional and structural implications. This geometry would facilitate diffusion of quinone molecules between RCs and the bc_1 complex, especially if the RCs are localized so that the Q_B sites, located on the M subunit, face the open side of the LH1 ring. Organization of LH1 complexes in C-shaped structures may also account for certain spectroscopic observations. Inhomogeneous absorption of LH1 complexes has been demonstrated by different approaches. Approximately 10% of the Bchl molecules of the LH1 complexes absorb at a long wavelength (Bchl 896) (Kramer *et al.*, 1984) and present a high value (0.25) of time-independent anisotropy (van Grondelle *et al.*, 1987, 1994; Hunter *et al.*, 1989). This minor fraction of the Bchl molecules was suggested to act as a trap for the excitation energy (van Grondelle *et al.*, 1994). One possible interpretation is that the two Bchl molecules localized at the extremities of the C-shaped structure correspond to this long wavelength form of LH1 and are a privileged site of energy transfer between the LH1 complexes and the RC.

Several lines of evidence favor a model in which the formation of the C-shaped structures of LH1 we observed are related to the presence of the PufX polypeptide. The PufX protein is a small hydrophobic polypeptide found so far only in *Rb.capsulatus* and *Rb.sphaeroides*. The presence of this polypeptide is essential for photosynthetic growth (Farchaus *et al.*, 1990; Lilburn *et al.*, 1992) and facilitates the exchange of quinone molecules between the Q_o site of the bc_1 complex and the Q_B site of the RC (Farchaus *et al.*, 1990; Lilburn *et al.*, 1992; Barz *et al.*, 1995a,b). The evidence for its involvement in the formation of C-shaped structures of LH1 can be summarized as follows: (i) deletion of the *pufX* gene induces a significant increase in the ratio of Bchl molecules to RC in both *Rb.capsulatus* and *Rb.sphaeroides* species (Farchaus *et al.*,

1992; Westerhuis *et al.*, 1993; McGlynn *et al.*, 1996; Francia *et al.*, 1998); (ii) low concentrations of the PufX protein inhibit the *in vitro* reconstitution of LH1 complexes for both *Rb.sphaeroides* and *Rb.capsulatus* (Recchia *et al.*, 1998). This small polypeptide may also be an essential factor for the supramolecular organization of the photosynthetic apparatus because the presence of dimeric LH1–RC complexes depends strictly on the presence of PufX (Francia *et al.*, 1998).

Our results suggest that the bc_1 complex occurs as a monomer in the tubular membrane of *Rb.sphaeroides*. Indeed, if the bc_1 complex were present in a dimeric form, this dimer would have to be localized between two S-shaped structures to fulfill the 1:2 ratio of bc_1 complex to RC. Thus, the unit cell would contain four RCs and be twice as large as observed. Our proposal of a monomeric bc_1 complex is at odds with several lines of biochemical and structural evidence in favor of a dimeric form of this complex. First, the bc_1 complex isolated from *Rb.sphaeroides* migrates as a dimer on sizing gels (Guergova-Kuras *et al.*, 1998). Secondly, a symmetric dimer is also present in the crystal structure of the mitochondrial bc_1 complex described recently at 2.9 Å resolution (Xia *et al.*, 1997; Zhang *et al.*, 1998). This dimeric association certainly stabilizes the isolated purified bc_1 complex. In a native membrane, however, specific interactions between the bc_1 complex and other membrane proteins involved in the formation of supercomplexes may also stabilize its monomeric form. This is the case, for example, for the supercomplex of bc_1 complex and cytochrome oxidase, in the 1:1 ratio, isolated and purified from the thermophilic bacterium PS3 (Sone *et al.*, 1987). The apparent molecular mass of this supercomplex (380 kDa; Sone *et al.*, 1987), which includes contributions of protein, lipid and detergent molecules, appears too low to accommodate two bc_1 complexes and two cytochrome oxidase complexes, but is consistent with the mass of a monomeric bc_1 plus a cytochrome oxidase complex. Similarly, the presence of the bc_1 complex in the native membrane of *Rb.sphaeroides* as a monomer rather than a dimer may result from the supramolecular organization of the photosynthetic chain. This supramolecular association may involve the subunit IV of the bc_1 complex. This subunit (14 kDa) has been found so far only in the isolated bc_1 complex of *Rb.sphaeroides* in addition to the three redox subunits, cyt *b*, cyt c_1 and Rieske protein (FeS) (Chen *et al.*, 1994). Several results suggest that this subunit is essential for photosynthetic electron transfer activity. Mutants deleted in subunit IV can grow only under phototrophic conditions after a period of adaptation of 2 days (Chen *et al.*, 1994). Moreover, the subunit IV is absent from aerobically grown cells of *Rb.sphaeroides* although the bc_1 complex is fully functional for respiration (Chen *et al.*, 1994).

We intend to analyze by cryo-electron microscopy the tubular membranes characterized here. This is expected to provide a more highly resolved picture of the mode of association of the different membrane complexes involved in bacterial photosynthesis, in particular the exact positioning of the bc_1 complex. Ultrastructural analysis of membranes isolated from mutants of *Rb.sphaeroides* deleted in *pufX* (McGlynn *et al.*, 1996) and/or subunit IV of the bc_1 complex may also help clarify the mechanisms

involved in supercomplex formation. It will also be important to determine, by detailed electron microscopic studies of native membranes of several other species of photosynthetic bacteria, whether the organization in C-shaped structures of the LH1 complexes of *Rb.sphaeroides* is restricted to this species, or is a more general feature allowing an efficient diffusion of quinone molecules between the RCs and the bc_1 complex.

Materials and methods

Growth of *Rb.sphaeroides*

Rb.sphaeroides forma sp. *denitrificans* was grown under anaerobic heterotrophic conditions using Hutner medium in the presence of 200 mM potassium nitrate as described previously (Sabaty *et al.*, 1994). Mutant RCLH10 of *Rb.sphaeroides* deleted in LH2 complexes was grown photosynthetically as described by Hunter *et al.* (1988). In both cases, cells were harvested in the late exponential phase and washed with 50 mM Tris–HCl pH 8, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF).

Purification of the tubular membranes

The photoheterotrophically grown cells were suspended in 50 mM Tris–HCl pH 8, 1 mM AEBSF, 15% (w/v) sucrose, 1 mg/ml lysozyme and 2 mM EDTA. After 30 min incubation at 30°C, a few crystals of DNase I and MgCl₂ were added and spheroplasts were disrupted by dilution in 50 mM Tris–HCl pH 8, 1 mM AEBSF and the intact spheroplasts and cells spun down at 4000 g. The tubular membranes were recovered in the supernatants of successive spins at 5000–20 000 g. In a final step, the supernatant was dialyzed against 50 mM Tris–HCl pH 8, 1 mM AEBSF, deposited on a 20–40% sucrose-step gradient and spun at 20 000 g for 10 min (Beckman, model L8-80M, SW41 rotor). The purified tubular membranes were recovered in the pellet.

Analytical methods

Gel electrophoresis. Polypeptide contents of purified membranes were determined according to Laemmli (1970), except that sodium dodecyl sulfate (SDS) was omitted from the gel and from the lower electrode buffer. Membrane proteins were first reduced by 2% β-mercaptoethanol and then denatured at 50°C for 10 min in the presence of 2% SDS (Ljungdall *et al.*, 1987). The solubilized samples then underwent polyacrylamide gel electrophoresis with a Mini Protean II apparatus (Bio-Rad). The resolving gel containing 15% acrylamide was stained with Coomassie Blue.

Absorption spectroscopy. Room temperature absorption spectra were recorded on an Hitachi U-2000 spectrophotometer. Low temperature spectra (77 K) were collected using a Varian Cary E5 double-beam scanning spectrophotometer equipped with a flow cryostat (Air Liquide, Grenoble) cooled with liquid nitrogen. The samples were oxidized with potassium ferricyanide (25 mM), and reduced with potassium dithionite (10 mM) or sodium ascorbate (10 mM).

Determination of RC concentration. RC concentrations were calculated from light-induced measurements performed at 603 and 542 nm with a laboratory-built apparatus (Joliot *et al.*, 1989) assuming a $\Delta\epsilon_{603-542} = 30/\text{mM}/\text{cm}$ (Dutton *et al.*, 1975).

Determination of the bc_1 concentration. The bc_1 complex concentration was determined from dithionite-reduced minus ascorbate-reduced difference spectra recorded in the 530–580 nm range and assuming a $\Delta\epsilon_{560-574}$ of 28/mM/cm (Berden and Slater, 1970).

Determination of the Bchl concentration. The Bchl molecules were extracted from the membranes using the method described by van der Rest and Gingras (1974). A suspension of purified tubes (2 ml, $A_{870} = 1.5$ –2) was lyophilized and extracted three times with small volumes of an acetone-methanol mixture (7:2 v/v). The pooled extracts were spun for 5 min at 4000 g. The absorption of the supernatant, adjusted to 10 ml, was measured at 771 nm to determine the concentration of Bchl using the method described by Clayton (1963).

Electron microscopy and image processing

Freeze-drying. After rapid cryofixation in liquid nitrogen, the quenched sample was mounted in a Cryofract (Reichert-Jung) and kept at –170°C under a vacuum $<10^{-7}$ Torr to avoid contamination. Sublimation was achieved by slowly increasing the sample temperature to –80°C over

~2 h. To avoid any contamination of the surface and improve the local vacuum, the sample was protected by a cold shroud during etching.

The replica surface exposed by etching was obtained by evaporation of platinum-carbon at a low incident angle (20°). To strengthen the replica, a 10–20 nm thick layer of carbon was evaporated onto the surface at an incident angle of 90°. Following the cleaning, the replicas were examined under a Philips CM 120 electron microscope.

Negative staining. Samples negatively stained with 1% (w/w) uranyl acetate were examined under a Philips CM120 electron microscope operating at 120 kV. Low-dose electron micrographs were recorded at a magnification of 45 000 on Kodak SO163 film developed in full-strength Kodak D-19 developer.

Image analysis. Micrographs were evaluated for focus, astigmatism and drift by optical diffraction. The selected area of the best images was digitized on a Leafscan 45S CCD-array microdensitometer (Leaf System, Inc.). The sampling was 0.4 nm on the specimen scale. Initial rectangular areas were padded up to 1024 or 2048 pixel squares. Lattice parameters were first determined using Spectra (Schmid *et al.*, 1993), then further processed using the MRC image analysis package (Crowther *et al.*, 1996). The projection map was calculated after merging data for several tubular membranes.

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References

- Adir, N., Axelrod, H.L., Beroza, P., Issacson, R.A., Rongey, S.H., Okamura, M.Y. and Feher, G. (1996) Co-crystallization and characterization of the photosynthetic reaction center-cytochrome c_2 complex from *Rhodobacter sphaeroides*. *Biochemistry*, **35**, 2535–2547.
- Allen, J.P., Feher, G., Yeates, T.O., Rees, D.C., Deisenhofer, J., Michel, H. and Huber, R. (1986) Structural homology of the reaction centers from *Rb. sphaeroides* and *Rp. viridis* as determined by X-ray diffraction. *Proc. Natl Acad. Sci. USA*, **83**, 8589–8593.
- Barz, W.P., Francia, F., Venturoli, G., Melandri, B.A., Verméglio, A. and Oesterheld, D. (1995a) The role of the *pufX* protein in photosynthetic growth of *Rhodobacter sphaeroides*. *PufX* is required for light-driven electron transfer and photophosphorylation under anaerobic conditions. *Biochemistry*, **34**, 15235–15247.
- Barz, W.P., Verméglio, A., Francia, F., Venturoli, G., Melandri, B.A. and Oesterheld, D. (1995b) The role of the *pufX* protein in photosynthetic growth of *Rhodobacter sphaeroides*. *PufX* is required for efficient ubiquinone/ubiquinol exchange between the reaction center QB site and the cytochrome bc_1 complex. *Biochemistry*, **34**, 15248–15258.
- Berden, J.A. and Slater, E.C. (1970) The reaction of antimycin with a cytochrome *b* preparation active in reconstitution of the respiratory chain. *Biochim. Biophys. Acta*, **216**, 237–249.
- Boonstra, A.F., Germeroth, L. and Boekema, E.J. (1994) Structure of the light-harvesting antenna from *Rhodospirillum rubrum* studied by electron microscopy. *Biochim. Biophys. Acta*, **1184**, 227–234.
- Chen, Y.R., Usui, S., Yu, C.A. and Yu, L. (1994) Role of subunit IV in the cytochrome bc_1 complex from *Rhodobacter sphaeroides*. *Biochemistry*, **33**, 10207–10214.
- Clayton, R.K. (1963) Absorption spectra of photosynthetic bacteria and their chlorophylls. In Gest, H., San Pietro, A. and Vernon, L.P. (eds), *Bacterial Photosynthesis*. Antioch Press, Yellow Springs, OH, pp. 495–500.
- Crofts, A.R., Guergova-Kuras, M. and Hong, S. (1998) Chromatophores heterogeneity explains phenomena seen in *Rhodobacter sphaeroides* previously attributed to supercomplexes. *Photosynth. Res.*, **55**, 357–362.
- Crowther, R.A., Henderson, R. and Smith, J.M. (1996) MRC image processing programmes. *J. Struct. Biol.*, **116**, 9–16.
- Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Structure of the proteins subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3 Å resolution. *Nature*, **318**, 618–624.
- Drews, G. and Golecki, J.R. (1995) Structure, molecular organization and biosynthesis of membranes of purple bacteria. In Blankenship, R.E., Madigan, M.T. and Bauer, C.E. (eds), *The Anoxygenic Photosynthetic Bacteria*. Kluwer, Dordrecht, The Netherlands, pp. 231–257.
- Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Cytochrome c_2 and reaction center of *Rhodopseudomonas sphaeroides* Ga membranes. Extinction coefficients, content, half-reduction potentials, kinetics and electric field alterations. *Biochim. Biophys. Acta*, **387**, 536–556.
- Engelhardt, H., Engel, A. and Baumeister, W. (1986) Stoichiometric model of the photosynthetic unit of *Ectothiorhodospira halochloris*. *Proc. Natl Acad. Sci. USA*, **83**, 8972–8976.
- Ermler, U., Fritzsche, G., Buchanan, S.K. and Michel, H. (1994) Structure of the photosynthetic reaction centre from *Rhodobacter sphaeroides* at 2.65 Å resolution: cofactors and protein-cofactor interactions. *Structure*, **2**, 925–936.
- Farchaus, J.W., Grünberg, H. and Oesterheld, D. (1990) Complementation of a reaction centre-deficient *Rhodobacter sphaeroides* *pufLMX* deletion strain in *trans* with *puf BALM* does not restore the photosynthetic-positive phenotype. *J. Bacteriol.*, **172**, 977–985.
- Farchaus, J.W., Barz, W.P., Grünberg, H. and Oesterheld, D. (1992) Studies on the expression of the *pufX* polypeptide and its requirement for photoheterotrophic growth in *Rhodobacter sphaeroides*. *EMBO J.*, **11**, 2779–2788.
- Fernández-Velasco, J.G. and Crofts, A.R. (1991) Complex or supercomplex: inhibitor titration shows that electron transfer in chromatophores from *Rhodobacter sphaeroides* involves a dimeric UQH₂: cytochrome c_2 oxidase and is delocalized. *Biochem. Soc. Trans.*, **19**, 588–593.
- Francia, F., Wang, J., Venturoli, G., Melandri, B.A., Barz, W.P. and Oesterheld, D. (1998) The *pufX* protein is involved in pseudo-dimerisation of reaction center-antenna core complexes in *Rhodobacter sphaeroides*. *Biochemistry*, in press.
- Ghosh, R., Ghosh-Eicher, S., DiBerardino, M. and Bachofen, R. (1994) Isolation and characterisation of a B875 protein kinase from *Rhodospirillum rubrum*. *Biochim. Biophys. Acta*, **1184**, 28–36.
- Golecki, J.R., Ventura, S. and Oelze, J. (1991) The architecture of unusual membrane tubes in the B800–850 light-harvesting bacteriochlorophyll-deficient mutant 19 of *Rhodobacter sphaeroides*. *FEMS Microbiol. Lett.*, **77**, 335–340.
- Guergova-Kuras, M., Salcedo-Hernandez, R., Bechmann, G., Kuras, R., Gennis, R.B. and Crofts, A.R. (1998) Expression and one-step purification of a fully active poly-histidine tagged *bc_1* complex from *Rhodobacter sphaeroides*. *Protein Expr. Purif.*, in press.
- Hankamer, B., Barber, J. and Boekema, E.J. (1997) Structure and membrane organization of photosystem II in green plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **48**, 641–671.
- Hunter, C.N., Pennoyer, J.D., Sturgis, J.N., Farrelly, D. and Niederman, R.A. (1988) Oligomerization states and associations of light-harvesting pigment-protein complexes of *Rhodobacter sphaeroides* as analysed by lithium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry*, **27**, 3459–3467.
- Hunter, C.N., van Grondelle, R. and van Dorssen, R.J. (1989) Construction and properties of M2192, a mutant of *Rhodobacter sphaeroides*, with the B875 antenna as the sole pigment protein. *Biochim. Biophys. Acta*, **973**, 383–389.
- Ikeda-Yamasaki, I., Odahara, T., Mitsuoka, K., Fujiyoshi, Y. and Murata, K. (1998) Projection map of the reaction center-light harvesting 1 complex from *Rhodopseudomonas viridis* at 10 Å resolution. *FEBS Lett.*, **425**, 505–508.
- Joliot, P., Verméglio, A. and Joliot, A. (1989) Evidence for supercomplexes between reaction centers, cytochrome c_2 and cytochrome bc_1 complex in *Rhodobacter sphaeroides* whole cells. *Biochim. Biophys. Acta*, **975**, 336–345.
- Joliot, P., Verméglio, A. and Joliot, A. (1990) Electron transfer between primary and secondary donors in *Rhodospirillum rubrum*: Evidence for a dimeric association of reaction centers. *Biochemistry*, **29**, 4355–4361.
- Joliot, P., Verméglio, A. and Joliot, A. (1996) Supramolecular organization of the photosynthetic chain in chromatophores and cells of *Rhodobacter sphaeroides*. *Photosynth. Res.*, **48**, 291–299.
- Joliot, P., Joliot, A. and Verméglio, A. (1997) Photo-induced cyclic electron transfer operates in frozen cells of *Rhodobacter sphaeroides*. *Biochim. Biophys. Acta*, **1318**, 374–384.
- Karrasch, S., Bullough, P.A. and Gosh, R. (1995) The 8.5 Å projection

- map of the light-harvesting complex I from *Rhodospirillum rubrum* reveals a ring composed of 16 subunits. *EMBO J.*, **14**, 631–638.
- Kiley, P.J., Varega, A. and Kaplan, S. (1988) Physiological and structural analysis of light-harvesting mutants of *Rhodobacter sphaeroides*. *J. Bacteriol.*, **170**, 1103–1115.
- Kramer, H.J.M., Pennoyer, J.D., van Grondelle, R., Westerhuis, W.H.J., Niederman, R.A. and Ames, J. (1984) Low-temperature optical properties and pigment organization of the B875 light-harvesting bacteriochlorophyll–protein complex of purple photosynthetic bacteria. *Biochim. Biophys. Acta*, **767**, 335–344.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lavergne, J., Joliot, P. and Verméglio, A. (1989) Partial equilibration of photosynthetic electron carriers under weak illumination: a theoretical and experimental study. *Biochim. Biophys. Acta*, **975**, 346–354.
- Lilburn, T.G., Haith, C.E., Prince, R.C. and Beatty, J. (1992) Pleiotropic effects of *pufX* gene deletion on the structure and function of the photosynthetic apparatus of *Rhodobacter capsulatus*. *Biochim. Biophys. Acta*, **1100**, 160–170.
- Ljungdall, P.O., Pennoyer, J.D., Robertson, D.E. and Trumpower, B.L. (1987) Purification of highly active cytochrome *bc*₁ complexes from phylogenetically diverse species by a single chromatographic procedure. *Biochim. Biophys. Acta*, **891**, 227–241.
- McDermott, G., Prince, S.M., Freer, A.A., Hawthornthwaite-Lawless, A.M., Papiz, M.Z., Cogdell, R.J. and Isaacs, N.W. (1995) Crystal structure of an integral membrane light-harvesting complex from photosynthetic bacteria. *Nature*, **374**, 517–521.
- McGlynn, P., Westerhuis, W.H.J., Jones, M.R. and Hunter, C.N. (1996) Consequences for the organization of reaction center-light harvesting antenna 1 (LH1) core complexes of *Rhodobacter sphaeroides* arising from deletion of amino acid residues from the C terminus of the LH1 α polypeptide. *J. Biol. Chem.*, **271**, 3285–3292.
- Meyer, T.E. and Donohue, T.J. (1995) Cytochromes, iron-sulfur and copper proteins mediating electron transfer from the cyt *bc*₁ complex to photosynthetic reaction center complexes. In Blankenship, R.E., Madigan, M.T. and Bauer, C.E. (eds), *The Anoxygenic Photosynthetic Bacteria*. Kluwer, Dordrecht, The Netherlands, pp. 725–745.
- Miller, K.R. (1982) Three-dimensional structure of a photosynthetic membrane. *Nature*, **300**, 53–55.
- Recchia, P.A., Davis, C.M., Lilburn, T.G., Beatty, J.T., Parkes-Loach, P.S., Hunter, C.N. and Loach, P.A. (1998) Isolation of the *pufX* protein from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*: Evidence for its interaction with the α -polypeptide of the core light-harvesting complex. *Biochemistry*, **37**, 11055–11063.
- Rögner, M., Boekema, E.J. and Barber, J. (1996) How does photosystem 2 split water? The structural basis of efficient energy conversion. *Trends Biochem. Sci.*, **21**, 44–49.
- Sabaty, M., Jappé, J., Olive, J. and Verméglio, A. (1994) Organisation of electron transfer components in *Rhodobacter sphaeroides* forma sp. *denitrificans* whole cells. *Biochim. Biophys. Acta*, **1187**, 313–323.
- Schmid, M.F., Dargahi, R. and Tam, M.W. (1993) SPECTRA: a system for processing electron images of crystals. *Ultramicroscopy*, **48**, 251–264.
- Sone, N., Sekimachi, M. and Kutoh, E. (1987) Identification and properties of a quinol oxidase super-complex composed of a *bc*₁ complex and cytochrome oxidase in the thermophilic bacterium PS3. *J. Biol. Chem.*, **262**, 15386–15391.
- Stahlberg, H., Dubochet, J., Vogel, H. and Ghosh, R. (1998) The reaction centre of the photounit of *Rhodospirillum rubrum* is anchored to the light-harvesting complex with 4-fold rotational disorder. *Photosynth. Res.*, **55**, 363–368.
- Stark, W., Kühlbrandt, W., Wildhaber, I., Wehrli, E. and Mühlethaler, K. (1984) The structure of the photoreceptor unit of *Rhodospseudomonas viridis*. *EMBO J.*, **3**, 777–783.
- Tiede, D.M., Vashista, A.C. and Gunner, M.R. (1993) Electron-transfer kinetics and electrostatic properties of the *Rhodobacter sphaeroides* reaction center and soluble *c*-cytochromes. *Biochemistry*, **32**, 4515–4531.
- van der Berg, W.H., Prince, R.C., Bashford, C.L., Takamiya, K., Bonner, W.D. and Dutton, P.L. (1979) Electron and proton transport in the ubiquinone cytochrome *b-c*₂ oxidoreductase of *Rhodospseudomonas sphaeroides*. Patterns of binding and inhibition by antimycin. *J. Biol. Chem.*, **254**, 8594–8604.
- van der Rest, M. and Gingras, G. (1974) The pigment complement of the photosynthetic reaction center isolated from *Rhodospirillum rubrum*. *J. Biol. Chem.*, **249**, 6446–6453.
- van Grondelle, R., Bergström, H., Sundström, V. and Gillbro, T. (1987) Energy transfer within the bacteriochlorophyll antenna of purple bacteria at 77 K, studied by picosecond absorption recovery. *Biochim. Biophys. Acta*, **894**, 313–326.
- van Grondelle, R., Dekker, J.P., Gillbro, T. and Sundström, V. (1994) Energy transfer and trapping in photosynthesis. *Biochim. Biophys. Acta*, **1187**, 1–65.
- Verméglio, A., Joliot, P. and Joliot, A. (1993) The rate of cytochrome *c*₂ photooxidation reflects the subcellular distribution of reaction centers in *Rhodobacter sphaeroides* Ga cells. *Biochim. Biophys. Acta*, **1183**, 352–360.
- Walz, T. and Ghosh, R. (1997) Two-dimensional crystallization of the light-harvesting I-reaction centre photounit from *Rhodospirillum rubrum*. *J. Mol. Biol.*, **265**, 107–111.
- Westerhuis, W.H.J., Farchaus, J.W. and Niederman, R.A. (1993) Altered spectral properties of the B875 light-harvesting pigment-protein complex in a *Rhodobacter sphaeroides* mutant lacking *pufX*. *Photochem. Photobiol.*, **58**, 460–463.
- Xia, D., Yu, C.A., Kim, H., Xia, J.Z., Kachurin, A.M., Zhang, L., Yu, L. and Deisenhofer, J. (1997) Crystal structure of the cytochrome *bc*₁ complex from bovine heart mitochondria. *Science*, **277**, 60–66.
- Zhang, Z., Huang, L., Shulmeister, V.M., Chi, Y.-I., Kim, K.K., Hung, L.-W., Crofts, A.R., Berry, E.A. and Kim, S.-H. (1998) Electron transfer by domain movement in cytochrome *bc*₁. *Nature*, **392**, 677–684.

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