

The Reaction Center Complex from the Green Sulfur Bacterium *Chlorobium tepidum*: A Structural Analysis by Scanning Transmission Electron Microscopy

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The three-dimensional (3D) structure of the reaction center (RC) complex isolated from the green sulfur bacterium *Chlorobium tepidum* was determined from projections of negatively stained preparations by angular reconstitution. The purified complex contained the PscA, PscC, PscB, PscD subunits and the Fenna-Matthews-Olson (FMO) protein. Its mass was found to be 454 kDa by scanning transmission electron microscopy (STEM), indicating the presence of two copies of the PscA subunit, one copy of the PscB and PscD subunits, three FMO proteins and at least one copy of the PscC subunit. An additional mass peak at 183 kDa suggested that FMO trimers copurify with the RC complexes. Images of negatively stained RC complexes were recorded by STEM and aligned and classified by multivariate statistical analysis. Averages of the major classes indicated that different morphologies of the elongated particles (length = 19 nm, width = 8 nm) resulted from a rotation around the long axis. The 3D map reconstructed from these projections allowed visualization of the RC complex associated with one FMO trimer. A second FMO trimer could be correspondingly accommodated to yield a symmetric complex, a structure observed in a small number of side views and proposed to be the intact form of the RC complex.

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Introduction

The P840-reaction center (RC) of *Chlorobium* belongs to the Fe-S type (type 1) which reduces Fe-S clusters, like the P700-RC of photosystem I (PSI) in oxygenic photosynthetic organisms, as well as the P800-RC of *Heliobacteria* (Blankenship, 1992; Feiler & Hauska, 1995). The similarity between the RC complexes of PSI, *Heliobacteria* and *Chlorobiaceae* has been substantiated by sequencing the genes for the RC core polypeptides *pscA* (for nomenclature, see Bryant, 1994) of *Chlorobium* (Büttner *et al.*, 1992;

Liebl *et al.*, 1993). However, based on the presence of only a single gene for the core proteins, a homodimeric core structure has been proposed for *Helio-bacteria* and *Chlorobiaceae* (Büttner *et al.*, 1992; Liebl *et al.*, 1993), in contrast to the heterodimeric core PsaA/PsaB of PSI. A recent structural analysis of the RC core complex from *Chlorobium tepidum* is consistent with a homodimer formed from two 82 kDa PscA proteins (Tsiotis *et al.*, 1997). Analogous to the PsaA/PsaB heterodimer of PSI, the homodimer of PscA of *Chlorobium* carries the primary donor (P840, a special pair of BChl_a), the primary electron acceptor A₀ (a monomeric Chl_a-derivative), an intermediate electron acceptor A₁, identified as menaquinone (Kjaer *et al.*, 1994; see Frankenbeg *et al.*, 1996), and the Fe-S cluster X (Feiler & Hauska, 1995). The PscA homodimer binds considerably less chlorophyll molecules (16 BChl_a molecules) in comparison to the PsaA/PsaB heterodimer (about 80 Chl_a), which is in line with

Abbreviations used: BChl_a, bacteriochlorophyll *a*; RC, reaction center; FMO, Fenna-Matthews-Olson; FMN, flavin mononucleotide; PscA-D, *Chlorobium* RC subunits; STEM, scanning transmission electron microscopy; PSI, photosystem I.

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comparatively few histidine residues in PscA (Griesbeck *et al.*, 1998). The gene *pscA* in *Chlorobia* is followed by a second gene *pscB* in a transcription unit (Büttner *et al.*, 1992). This second gene codes for a 23 kDa protein with two Fe-S clusters, analogous to the terminal electron acceptors F_A and F_B of PSI which are located on the *psaC* gene product (Golbeck & Bryant, 1991). An additional protein of the *Chlorobia* RC complex, denoted PscD (16.5 kDa), is thought to be the analog of the PsaD, PSI protein (Hager-Braun *et al.*, 1995). Similar to PSI which binds the electron donor plastocyanin or cytochrome *c*₅₅₃, the RC of green sulfur bacteria binds the 23 kDa protein, cyt *c*₅₅₁ (*pscC* gene product) to reduce P₈₄₀⁺ (Hurt & Hauska, 1984; Kusumoto *et al.*, 1994; Oh-Oka *et al.*, 1993; Okkels *et al.*, 1992). Finally, it is not clear whether the 23 kDa PscC protein is present as a monomer or as a dimer in the complex (Feiler *et al.*, 1992; Kusumoto *et al.*, 1994; Okkels *et al.*, 1992).

In preparations capable of stable charge separation, the PscA protein is associated with a 40 kDa polypeptide that carries 7 BChl_a, known as the Fenna-Matthews-Olson (FMO) protein (Matthews *et al.*, 1979). This protein is not required for the primary charge separation to occur, but seems to stabilize the RC (Feiler *et al.*, 1992; Hager-Braun *et al.*, 1995; Oh-Oka *et al.*, 1993; Okkels *et al.*, 1992). The number of FMO proteins found in various RC preparations is not consistent (Feiler & Hauska, 1995). Recently, a re-evaluation of the FMO content based on the different extinction coefficients of the BChl_a molecules in the RC and in the FMO (Griesbeck *et al.*, 1998) gave a stoichiometry of one to two FMO trimers per RC in the functionally competent fraction.

Our procedure using Triton X-100 for the isolation of the P840-RC from *C. tepidum* yields a

lighter core complex banding as a brownish-green band at lower sucrose density, and a heavier functionally intact complex banding as a bluish-green band at higher sucrose density (Hager-Braun *et al.*, 1995). The brownish-green core complex has recently been analyzed by gel filtration and electron microscopy, revealing a PscA homodimer associated with a single PscC protein (Tsiotis *et al.*, 1997). On examination, the bluish-green fraction was found to contain the PscA, PscB, PscC, PscD and FMO proteins and showed steady-state, light-induced, electron transfer (Feiler & Hauska, 1995).

In the present communication we report the structural analysis of the complete RC complex present in the bluish-green fraction resulting from Triton X-100 solubilized preparations of *C. tepidum* membranes. Structural information was obtained by gel filtration, mass determination, as well as negative staining using the scanning transmission electron microscope (STEM) and image processing. Both the mass analysis and the three-dimensional (3D) low-resolution structure provided evidence for the presence of one firmly bound FMO trimer per purified RC complex, although the structure could accommodate two trimers. Accordingly, we propose the intact form of the RC to contain two FMO trimers.

Results

Characterization of the purified complexes by SDS-PAGE, HPLC gel filtration and absorption spectroscopy

The less dense brownish band has previously been shown to contain only PscA, PscC and traces of the FMO proteins (Hager-Braun *et al.*, 1995). On gel filtration the denser bluish-green band yielded

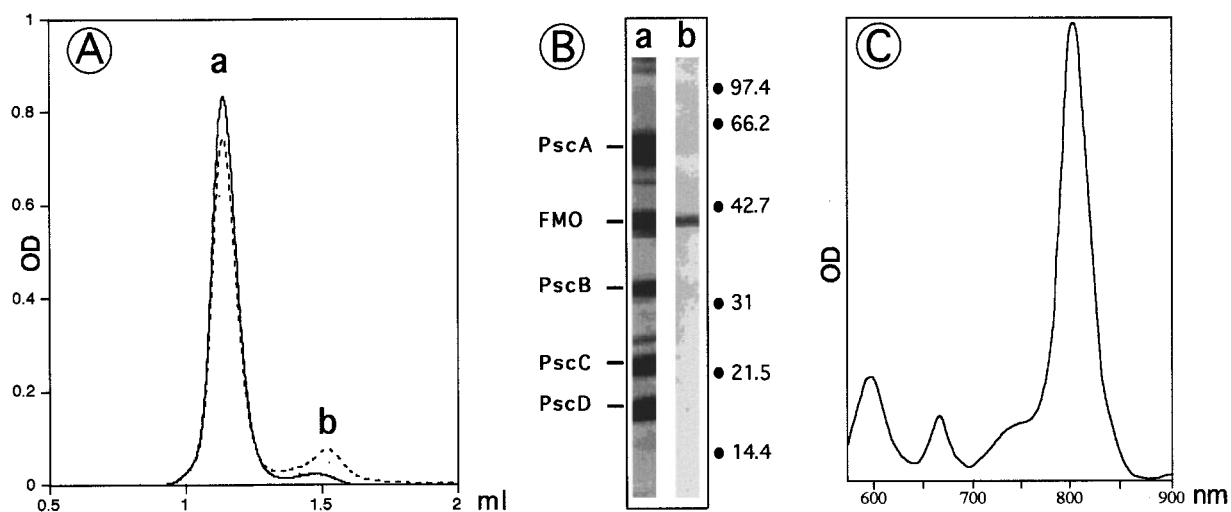


Figure 1. (a) Elution profile at 435 nm (continuous line) and 598 nm (broken line) of the bluish-green band in 10 mM Tris, 0.1% (v/v) Triton X-100 (pH 7.5) containing 150 mM NaCl. The flow rate was 0.05 ml/minute on a Sephadex 200 3.2/30 gel filtration column attached to a SMART system. The protein concentration was 1 mg/ml and the sample volume was 0.05 ml. (b) Silver stained SDS gels of the fraction a (lane a) and fraction b (lane b). (c) Absorption spectrum of the purified complex (fraction a).

a major and a minor fraction (Figure 1(a), peaks a and b, respectively). Both were analyzed by SDS gel electrophoresis (Figure 1(b)). The major fraction (peak a) exhibited protein bands at 55 kDa, 40 kDa, 32 kDa, 22 kDa and 16 kDa on the gel (lane a). The diffuse band at 55 kDa is the PscA protein, which has a calculated molecular mass of 82.5 kDa (Büttner *et al.*, 1992), but is known to run as a diffuse band at 55-60 kDa on SDS gels, similar to the PsaA and PsaB proteins of PSI (Golbeck & Bryant, 1991). The band at 22 kDa was identified as the PscC subunit by heme-staining (data not shown). The bands at 40 kDa, 32 kDa and 16 kDa are the FMO, the PscB and the PscD proteins, respectively (Hager-Braun *et al.*, 1995). Thus, all of the proteins forming the RC complex (PscA, PscB, PscC, PscD) and the FMO were present in the isolated complex. The minor gel filtration fraction (peak b) only contained a small amount of FMO (Figure 1(b), lane b). The absorption spectrum of the RC complex isolated in the major gel filtration fraction is shown in Figure 1(c). There are maxima at 810 nm, 671 nm (A_0 electron acceptor) and 600 nm (Q_x of the BChl a). The ratio of the absorbance at 810 nm to the shoulder at 835 nm is between four and five. This fraction was used to perform mass measurement and structural analysis by STEM.

Mass measurement of the purified reaction center complexes with the STEM

Mass analysis of freeze-dried unstained Triton X-100 solubilized RC complexes was performed with the STEM. Projections from particles of two different sizes could be visually distinguished on the images (Figure 2(a)), and were selected accordingly for the evaluation. The pooled mass data are shown in Figure 2(b). The histograms were described by two Gaussian curves, yielding peaks

Table 1. Calculated mass of the reaction center complex of *C. tepidum*

Gene products	Mass (kDa)
PscA	82.5 (2 \times)
PscB	23
PscC	23
PscD,	16.5
BChl a , Chl a	20
FMO	40 (3 \times)
BChl a	7 (3 \times)
Calculated	388
Measured (STEM)	454 \pm 74
Detergent/lipid	60

at 175(\pm 47) kDa ($n = 621$) and 435(\pm 73) kDa ($n = 2021$). Considering the imaging electron dose of 348(\pm 20) e/nm^2 , these values become 183(\pm 47) kDa and 454(\pm 74) kDa after correction for the experimentally measured beam-induced mass loss (data not shown). Comparison of the mass histograms and the SDS gels allows the mass of 454 kDa to be assigned to complexes which contain the PscA, PscB, PscC, PscD and the FMO proteins (Table 1). The additional mass peak at 183 kDa resulting from the smaller particles is close to the mass of one FMO trimer (Table 1).

Structural analysis of the reaction center complexes by STEM

STEM images of negatively stained RC complexes recorded at 100 kV revealed a rather heterogeneous population of smaller spherical, and larger elongated particles. The smaller particles often had a stain-filled central pit and sometimes a triangular appearance (Figure 3, arrowhead). Such particles have previously been identified as FMO trimers (Tsiotis *et al.*, 1997). The elongated particles were

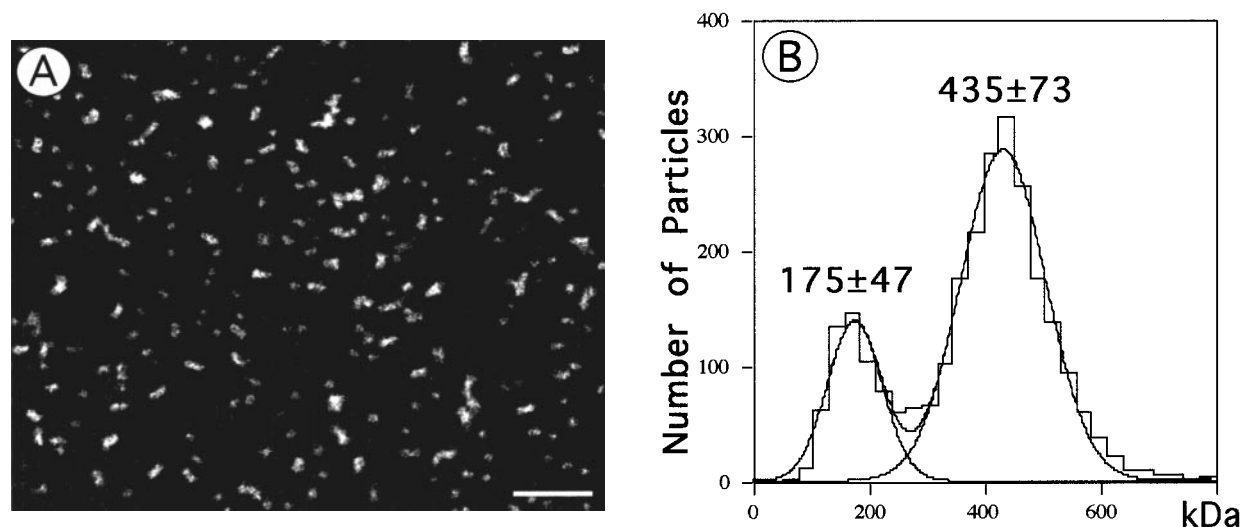


Figure 2. Mass analysis of the purified RC from *C. tepidum*. (a) STEM dark field image of unstained freeze-dried RC complexes. The scale bar represents 70 nm. (b) Mass histogram from the RC particles ($n = 2642$). The Gauss peaks fitted at 175(\pm 47) and 435(\pm 73) kDa, respectively. The imaging dose was 348(\pm 20) e/nm^2 .

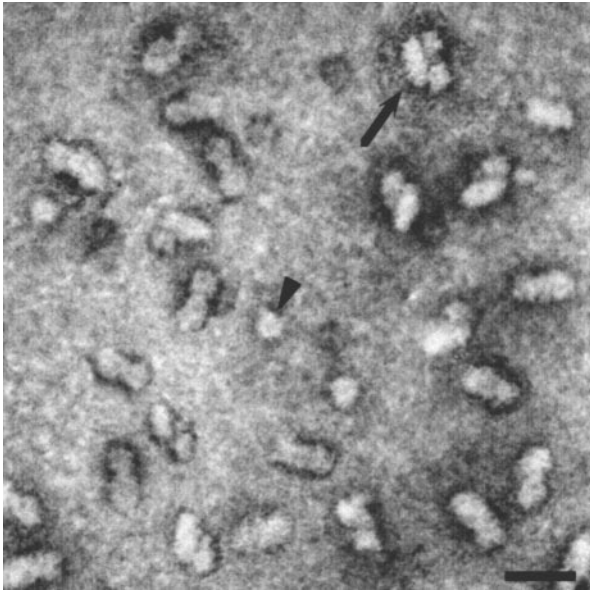


Figure 3. STEM dark field image of *C. tepidum* RC complexes. The contrast has been adapted to show the protein in light shades. The arrowhead indicates the small particles identified as FMO proteins, while the arrow indicates RC complexes with two associated FMO trimers. The scale bar represents 20 nm.

quite often asymmetric, as if they were associated with one of the smaller spherical particles. Infrequently, a second smaller particle was also attached, giving the complex mirror symmetry (Figure 3, arrow). A total of 4094 well-preserved elongated particles were aligned angularly and translationally, and submitted to a multivariate statistical analysis with subsequent classification, without imposing any symmetry. The classification grouped 64% of the images into nine major classes. Two classes containing 13% of the data set represent the side view of the complex (Figure 4(a) and (i)). Three classes containing 15% of the data set had a rather symmetric appearance and represent various top views of the complex (Figure 4(d), (e) and (f)). Four other classes (Figure 4(b), (c), (g) and (h)) containing 36% of the data set were mirror images of one another, and thus exhibited different handedness (compare Figure 4(b), (c) and (g), (h), respectively). Comparison with the side and the top view projections allows them to be classified as tilted side view projections, in accordance with the different handedness and the width variations observed. Averages of closely related, similar views were added to yield Figure 5(a) ($n = 551$, sum of Figure 4(a) and (i)), 5(b) ($n = 626$ sum of Figure 4(d), (e) and (f)), 5(c) ($n = 744$, sum of Figure 4(b) and (c)) and 5(d) ($n = 710$, sum of Figure 4(g) and (h)). From independent averages of the particles accumulated in the average displayed in Figure 5(b), the resolution values determined by the ring correlation function (Saxton & Baumeister, 1982), the phase residual

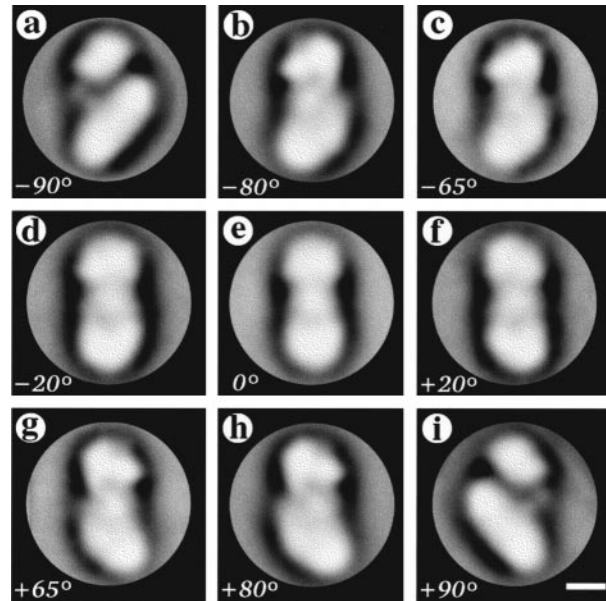


Figure 4. Averages from 4094 projections of negatively stained RC complexes which were aligned and classified by multivariate statistical analysis. A total of 64% of the images were decomposed into nine classes with (a) 301, (b) 633, (c) 111, (d) 233, (e) 242, (f) 151, (g) 164, (h) 546, and (i) 250 members, respectively. The scale bar represents 5 nm.

(Frank, 1975) and the spectral signal-to-noise ratio (Unser *et al.*, 1989) are 1.7 nm, 2.3 nm and 1.9 nm, respectively. The side view (Figure 5(a)) reveals the association of two protein domains. One is a

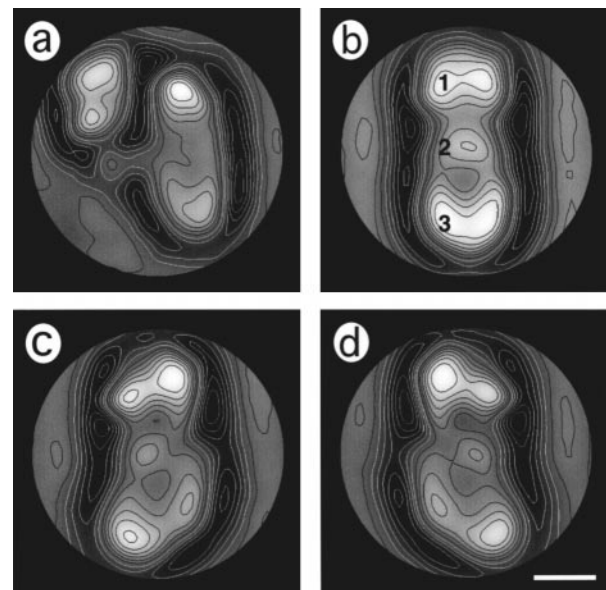


Figure 5. Four views of the RC complex. (a) Sum of 551 side view projections (see Figure 4(a) and (i)). (b) Sum of 626 top view projections (see Figure 4(d), (e) and (f)). (c) Sum of 744 tilted side view projections (see Figure 4(b) and (c)). (d) Sum of 710 tilted side view projections (see Figure 4(g) and (h)) with the opposite handedness to (c). The scale bar represents 5 nm.

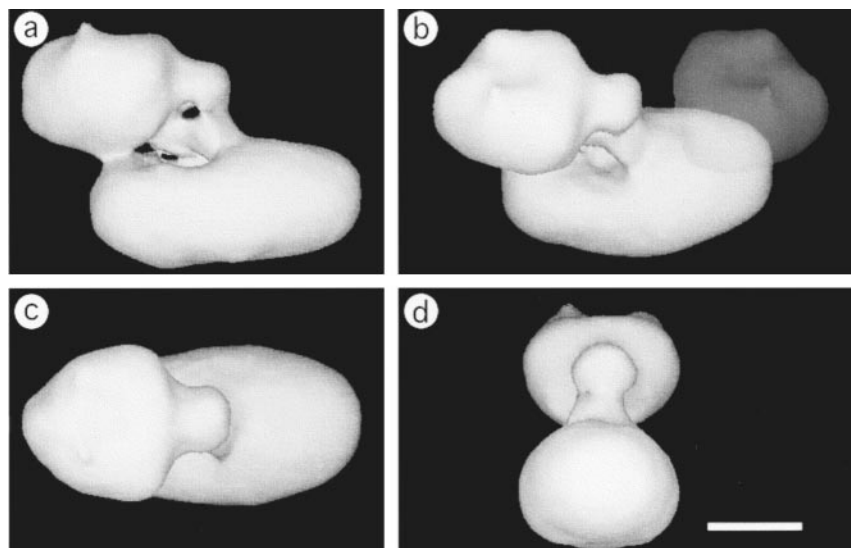


Figure 6. The 3D reconstruction of the RC complex. A total of 2741 images were used from the total data set of 4094. (b) The additional copy of an FMO trimer was placed to indicate its hypothetical location. The scale bar represents 5 nm.

15 nm long and 6 nm wide ellipsoid having two peripheral mass peaks. The other is more compact, about 8 nm \times 6 nm, and located on the periphery of the elongated domain. The two structures are linked by a protrusion emanating from the center of the complex. The entire structure has a length of 19 nm. The top view projection (Figure 5(b)) exhibits three protein domains (labeled as 1 to 3) and has a similar length of 18.0-19.0 nm and a width of 7.5-8.0 nm.

The mirror symmetric projections, tentatively assigned as projections of the complex rotated around its long axis, were both the same length as the top view projection and similarly exhibited three domains. However, their width and morphology were distinctly different. They were used as the initiator images for a 3D reconstruction using angular reconstruction as described in Materials and Methods. Repeated refinement yielded a final structure composed of an elongated subcomplex of 15 nm long, 8 nm wide and 5-6 nm thick, which had a compact, slightly triangular subcomplex 8 nm in diameter and 6 nm thick attached on one side through a central stalk emanating from the elongated unit (Figure 6). On contouring this structure to yield the total mass of 454 kDa which was measured by STEM, the triangular subcomplex was shown to have a mass of 145 kDa, compatible with the mass of an FMO trimer.

Discussion

Several solubilized, photoactive RC preparations from *Chlorobia* have been reported (for a review, see Feiler & Hauska, 1995) and the isolated complexes have been characterized by different spectroscopic methods such as absorption spectroscopy, EPR and FTIR (Feiler *et al.*, 1992, 1994; Kjaer *et al.*, 1994; Miller *et al.*, 1992; Nitschke *et al.*, 1990). A study of a RC subcomplex from the green sulfur bacterium *C. tepidum* containing the

PscA and PscC subunits has recently been reported (Tsiotis *et al.*, 1997). The mass of the complex determined by STEM (248 kDa), coupled with the dimensions (14 nm long \times 8 nm wide) and distinct asymmetry revealed by images recorded from negatively stained samples, indicated the association of a PscA homodimer with one copy of the PscC protein.

In the present work we describe the 3D structure of the complete RC complex from *C. tepidum* which contains the PscA, PscB, PscC, PscD and FMO subunits. This was prepared by the method described by Hager-Braun *et al.* (1995). While gel filtration indicated the presence of a homogeneous complex, STEM suggested at least two particle types. The mass of 454 kDa determined by STEM for the freeze-dried detergent-solubilized complex (Figure 2) is consistent with the presence of two copies of PscA (2×82.5 kDa), one copy of the PscB (23 kDa), PscC (23 kDa) and PscD (16.5 kDa) proteins, three FMO proteins (3×40 kDa) and 20 BChl*a* antenna molecules (20×1 kDa). Reference to the previously studied PscA/PscC subcomplex (Tsiotis *et al.*, 1997) allows the remaining 60 kDa to be assigned to associated detergent molecules. Although the latter value is indeed close to the mass of a Triton X-100 micelle of 62-96 kDa (Robson & Dennis, 1977), the presence of two PscC proteins indicated by spectroscopic analysis (Oh-Oka *et al.*, 1995) cannot be entirely excluded.

The population of smaller particles present on the STEM images has a mass of 183 kDa. Accordingly, they are most probably FMO trimers. In support of this assignment, small particles evident on images of negatively stained RC complexes often had a triangular projection (Figure 3, arrowhead). Thus, both methods indicate that free FMO trimers copurified with the RC complexes. A few side views from negatively stained RCs indicated the presence of two FMO trimers (see below). Also, the possibility cannot be excluded that some FMOs

dissociated from the RC complex during the staining step. Consequently, estimates of the RC-associated FMO based on absorption ratios, which have predicted the presence of three FMO trimers per RC (Franke *et al.*, 1997), may not be precise since this technique is not able to discriminate between FMO bound to the RC and FMO free in solution.

Multivariate statistical analysis of STEM dark field images from negatively stained specimens (Figure 3) revealed top and side views of the main population of elongated particles (Figure 4(a)-(i)). The height and the length of the elongated subcomplex evident in the side view average (Figure 5(a)) about 6 nm and 15 nm, respectively, correspond closely to the dimensions of the PscA/PscC core complex (Tsiotis *et al.*, 1997). Compatible with this assignment, high protein densities are located at both ends of the longitudinal axis, being separated by a distance of about 10 nm as in the PscA/PscC core complex. The width of the top view also agrees with the width of the monomeric PscA/PscC complex (Tsiotis *et al.*, 1997). A total of 2701 projection images were used to calculate a 3D map (Figure 6). In this 3D reconstruction the ellipsoid domain correlates with the elongated structure visible in projection. From its size, this domain is the membrane-associated part of the complex composed of the PscA homodimer, the BChla antenna molecules and the PscC subunit (Tsiotis *et al.*, 1997). The volume of the displayed structure (Figure 6) multiplied by $0.8 \text{ Da}/\text{\AA}^3$ gives a mass of 270 kDa for the RC and 145 kDa for the FMO trimer. The triangular subcomplex has both the form and dimensions (Figure 6(c)) of an FMO trimer (Li *et al.*, 1997; Matthews *et al.*, 1979). This assignment identifies the cytoplasmic side of the complex. As shown in Figure 6(a), the FMO trimer interacts directly with the periphery of the PscA homodimer, but also with a knob protruding from the membrane-bound elongated disk. The protruding structure is likely to represent the PscB and PscD proteins missing in the RC core complex analyzed previously (Tsiotis *et al.*, 1997).

In analogy to the 4 Å structure of the cyanobacterial PSI reaction center (Schubert *et al.*, 1997), the BChla antenna molecules of the *C. tepidum* RC complex are expected to form an oval cluster with a long axis of approximately 13 nm. Thus, the contact of the FMO trimer with the periphery of the elongated disk suggests energy transfer from the FMO to the primary electron donor, P840, *via* BChla. Further, the extrinsic location of the FMO trimer clearly revealed by the 3D reconstruction (Figure 6(a)) is in contrast to the hypothesis of a partially integrated FMO complex (Li *et al.*, 1997).

In analogy to the heterodimeric PSI complex, the presence of both the Fe-S cluster protein (PscB) and the putative ferredoxin docking protein (PscD) on the cytoplasmic side of the RC has been proposed. This was based on EPR measurements and sequence analysis (Büttner *et al.*, 1992; Hager-Braun *et al.*, 1995; Illinger *et al.*, 1993; Liebl *et al.*, 1993). As noted above, the knob protruding from

the membrane resident core complex is likely to be composed of the two additional proteins, PscB and PscD (Figure 6). Since PscD tends to associate with FMO trimers (Hager-Braun *et al.*, 1995; Tsiotis *et al.*, 1997), the protein in contact with the FMO trimer is probably the PscD subunit. Accordingly, the cytoplasmic protein which is located in the middle of the PscA homodimer is probably the PscB protein which contains the Fe-S cluster. This central location closely corresponds to the position of the related PsaC protein in the heterodimeric PSI complex of cyanobacteria (Krauß *et al.*, 1996).

A few side views on the images recorded from negatively stained preparations exhibited two peripheral particles associated with the elongated core complex. These projections probably arose from RCs with two FMO trimers. Indeed, as indicated in Figure 6(b), transparent domain, there would be space for the association of a second correspondingly attached FMO trimer. Thus, we propose the intact form of the RC to contain two FMO trimers. The structural analysis of RC complexes which, with certainty, contain the full complement FMO trimers is now required to finally resolve the organization of the antenna system in the RC of *Chlorobia*.

Materials and Methods

Analytical methods

The RC of *C. tepidum* was isolated as described (Hager-Braun *et al.*, 1995). Photosynthetic membranes were solubilized using Triton X-100. Anaerobic conditions were established by including FMN in all solutions and illuminated with white light before use. The extract was fractionated on a sucrose density gradient containing Triton X-100 and yielded a brownish and a bluish-green band. The latter denser band which contained the PscA, PscB, PscC, PscD and FMO proteins, was used for structural analysis. Gel electrophoresis was carried out as outlined by Tsiotis *et al.* (1993). The gels were stained with silver nitrate. A Superdex 200 PC 3.2/30 (Pharmacia) analytical molecular sieve column attached to a Pharmacia SMART system was used for gel filtration. The column was equilibrated with buffer A (10 mM Tris, 0.1 % Triton X-100, pH 7.5) at a flow rate of 0.05 ml/minute. The filtrations were performed using two wavelengths of light, 435 nm and 598 nm, to detect the eluted proteins. The column temperature was 8 °C.

Scanning transmission electron microscopy and image processing

Mass analysis of detergent-solubilized particles was carried out as described (Müller & Engel, 1998). The complexes were diluted (1:1000) with buffer A prior to grid preparation. Elastic dark field images of unstained purified complexes were recorded using a VG-HB5 STEM at defined electron doses and 80 kV acceleration voltage. The particle mass was evaluated by measuring the number of electrons elastically scattered by a circular region enclosing the particle and subtracting the background contribution due to the thin carbon film using the IMPSYS software (Müller *et al.*, 1992). Gaussian curves were fitted to the mass histogram peaks. In

addition, beam-induced mass loss was experimentally assessed by repeatedly scanning the same grid region and monitoring the mass values (Müller *et al.*, 1992).

Dark field images of negatively stained particles were recorded with the same instrument operated at 100 kV acceleration voltage. Particles sampled at 0.35 nm were selected interactively, using the SEMPER image processing system. The particles were boxed into 64×64 pixel frames and aligned by correlation methods (Frank, 1975). Subsequently, multivariate statistical analysis (Frank *et al.*, 1988; Harauz *et al.*, 1988; van Heel, 1984) was applied to classify the selected particles using an algorithm kindly provided by Dr J. P. Breaudiere. Closely related clusters were then combined to yield the final averages. In addition, the resolution was assessed using the ring correlation function (Saxton & Baumeister, 1982), the phase residual, and the spectral signal-to-noise ratio (Unser *et al.*, 1989).

Three-dimensional reconstruction of the RC complexes

Images of single particles were processed using the programs SPIDER and WEB (Frank *et al.*, 1981; Radermacher, 1988). The reference-free algorithm described by Penczek *et al.* (1992) was used for particle alignment. The images were classified using multivariate statistical analysis with Ward's method as clustering criterion (Frank *et al.*, 1988) and averages of each class were calculated. These class averages were used with manually assigned tilt angles to calculate a first 3D volume by backprojection, assigning the angles -90° , -80° , -65° , -20° , 0° , $+20^\circ$, $+65^\circ$, $+80^\circ$ and $+90^\circ$. This volume was projected in 720 evenly spaced directions perpendicular to its long axis to generate a series of projection images. Each of the 2701 boxed particle images was then aligned against all projection images of the 3D volume and the correlation coefficients were calculated as described above. The projection image with the highest correlation coefficient defined the refined tilt-angle of that individual particle image. Once the set of refined tilt-angles for all particle images had been obtained, a new 3D volume was calculated by backprojection. Iteratively this 3D volume was used as the new reference for the next refinement of the tilt angles, until the resulting 3D volume had converged to a stable structure. Variations of the starting conditions were chosen to ensure the reproducibility of the final volume; the initial angular assignment was varied and features of the reconstruction were masked out to check if during the repeated refinement they would reappear. The tilt axis was always assumed parallel with the long axis of the particle.

Acknowledgments

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