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Structural analysis of the photosynthetic reaction center from the green sulfur bacterium *Chlorobium tepidum*

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Abstract

The reaction center (RC) core complex was isolated from the green sulfur bacterium *Chlorobium tepidum* and characterized by gel electrophoresis, gel filtration, and analytical ultracentrifugation. The purified complex contained the PscA and PscC subunits and a small amount of the Fenna–Matthews–Olson protein (FMO protein) as an impurity. The mass of the core complexes was found to be 248 kDa by scanning transmission electron microscopy (STEM). This is compatible with the presence of two copies of the PscA subunit and at least one copy of the PscC subunit, and provides evidence that the isolated complex exists as a monomer. Digital images of negatively stained RC complexes were recorded by STEM and analyzed by single-particle averaging. The complex had a length of 14 nm and a width of 8 nm, comparable to the length and width of the monomeric cyanobacterial PSI complex. The averages revealed a pseudo two-fold symmetry axis, which is a prominent structural element of the monomeric form. © 1997 Elsevier Science B.V.

Keywords: Electron microscopy; Green sulfur bacteria; Reaction center

1. Introduction

Photosynthetic reaction centers (RCs) are classified according to the nature of their terminal electron acceptors. Q-type RCs (type II) reduce a mobile quinone. Examples are found in purple bacteria and in the photosystem II (PSII) of cyanobacteria and higher plants. Iron–sulphur RCs (type I) reduce Fe–S centers, and occur in photosystem I (PSI) and in green sulfur bacteria such as *Chlorobia* and in *Heliobacteria* [1]. The similarity between the PSI type I RCs of higher plants, green algae and cyanobacteria and the type I RC of *Chlorobia* and *Heliobacteria* has been amply documented [2–4]. The sequences of the genes coding for the large subunits (PscA and PshA with a mass of 82 kDa; see [5]) show their core to be composed of two identical transmembrane subunits which form a homodimer [6,7]. Analogous to the *Psa*A/*Psa*B heterodimer of PSI, the homodimer of PscA of the *Chlorobia* carries the primary electron donor P_{840} (a special pair of BChl *a*), the primary

Abbreviations: BChl *a*, bacteriochlorophyll *a*; FMO, Fenna–Matthews–Olson; Psc, gene coded *Chlorobia* subunits; RC, reaction center; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STEM, scanning transmission electron microscopy

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electron acceptor A_0 (a Chl *a* monomer), an intermediate acceptor A_1 (speculated to be a menaquinone, but see [8]) and an Fe–S cluster analogous to F_X of PSI. The PscA protein contains 20 histidine residues compared to 42 and 37 in PsaA and PsaB, respectively [6,7]. In analogy to PSI it was proposed that these are candidates for binding the BChl *a* molecules. For *Chlorobium limicola* a transcription unit has been identified which contains a second gene (*psc*B) downstream of that coding for the large core subunit. It corresponds to a 23 kDa protein which contains two Fe–S clusters, analogous to the terminal electron acceptor F_A and F_B of PSI which are located on the *psa*C gene product [9]. An additional protein, denoted PscD, has been detected in the *Chlorobia* RC complex. The gene coding for PscD has been sequenced and represents a 16.5 kDa polar protein [10] which is thought to be the analog of the PsaD protein. Similar to PSI which binds the electron donor plastocyanin or cyt_{553}, the RC of green sulfur bacteria binds cyt c_{551} (*psc*C gene product), a 20 kDa protein, to reduce P^+_{840} [11–14]. However, there is a controversy about the nature of this protein [12,14,15]. The association of the PscA protein with a 42 kDa protein (the FMO protein) has also been observed. This protein is not required for the primary charge separation but seems to stabilize the RC [10,12,13,15]. It carries 7 BChl *a* molecules and was the first chlorophyll protein structure to be solved by X-ray crystallography [16]. Although the FMO protein has been found in various RC preparations, the exact number of copies per RC is not known [4].

A purified preparation of the P_{840} RC from *Chlorobium* with only three subunits, has been obtained using the detergent octyl glucoside. This exhibited labile photoactivity [11]. Recently, several reports of the isolation of more efficient RCs from *Chlorobia* have been published [10,14,15,17,18]. *Chlorobium tepidum* RC complexes which show steady state, light induced, electron transfer contain the PscA, PscB, PscC, PscD and FMO proteins. Recently, the isolation of a subcomplex including the PscA and PscC proteins was reported which showed light induced charge separation and recombination between the P_{840} and the primary electron acceptor [19].

The structure of the Q-type RCs in purple bacteria has been elucidated at atomic resolution by X-ray crystallography [20,21]. Knowledge about Fe–S-type RCs is lagging behind, but the PSI RC which has a heterodimeric nature has been crystallized in 2D and 3D crystals [22–27], and structural resolution is in progress [28,29]. The arrangement of the core in green sulfur bacteria may aid a better understanding of RCs in general, in particular of their evolution and of the selection pressure for the formation of heterodimeric cores in aerotolerant organisms.

In the present communication, we report structural studies of a RC complex containing the PscA and PscC subunits from the green sulfur bacterium *Chlorobium tepidum*. Structural information was obtained by gel filtration, analytical ultracentrifugation, mass determination using the scanning transmission microscope (STEM), and image processing. The mass analysis provided solid evidence for the homodimeric nature of the RC complex in the green sulfur bacterium *Chlorobium tepidum*.

2. Materials and methods

2.1. Analytical methods

The RC of *Chlorobium tepidum* was isolated as described [10]. Photosynthetic membranes were solubilized using Triton X-100 under anaerobic conditions. The extract was fractionated on a sucrose density gradient containing Triton X-100 and yielded a brownish and a bluish green band. The brownish band which contained the PscA and PscC proteins, was used for structural analysis. Gel electrophoresis was carried out as outlined in [26] and 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 10 mM Tris, 0.1% NaN_3, 0.15% Triton X-100, pH = 8 (buffer A) at a flow rate of 0.1 ml/min. The filtrations were performed using two wavelengths of light, 435 and 598 nm, to detect the eluted proteins. The column temperature was 8°C. The sedimentation velocity of PscA/PscC/detergent particles was determined at 20°C with the Beckman model XLA analytical ultracentrifuge equipped with absorption optics. Sedimentation velocity runs were carried out at 52 000 rpm in a 12 mm double sector Epon cell.

2.2. Scanning transmission electron microscopy and image processing

Detergent solubilized particles were adsorbed to thin carbon films supported by thicker fenestrated carbon layers, washed on 3 droplets of quartz bi-distilled water and either freeze-dried without staining, or negatively stained with a 1% uranyl formate solution and air dried. For mass analysis, elastic dark field images of unstained 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 [30]. Gauss curves were fitted to the mass histogram peaks [30].

Dark-field images of negatively stained particles were recorded in the same instrument at 100 kV acceleration voltage. Well preserved particles sampled at 0.35 nm were selected interactively, using the SEMPER image processing system installed on a VAX 3100 work station. As a first step 64 × 64 pixel regions including RC complexes were selected. The particles were aligned using correlation techniques, starting with a single projection as first reference. Intermediate references were constructed from the sums of those particles whose correlation coefficients with the reference were above a threshold value. In each run, particles contributing to the final average were accumulated into two independent averages from which the resolution was assessed using the ring correlation function [31], the phase residual [32], and the spectral signal-to-noise ratio [33]. Subsequently, multivariate statistical analysis [34,35] was applied. After classification of the selected particles using an algorithm kindly provided by Dr. J.P. Bretaudiere [36] and implemented in IMPSYS, a dendrogram representing the likeness of the different clusters was obtained. Closely related clusters were then added to improve the signal-to-noise ratio of the final averages.

3. Results

3.1. Characterization of the purified complexes by SDS–PAGE, HPLC gel filtration and analytical ultracentrifugation

The RC complex was isolated from *Chlorobium tepidum* using Triton X-100 to solubilize the photosynthetic membranes. A subsequent sucrose density

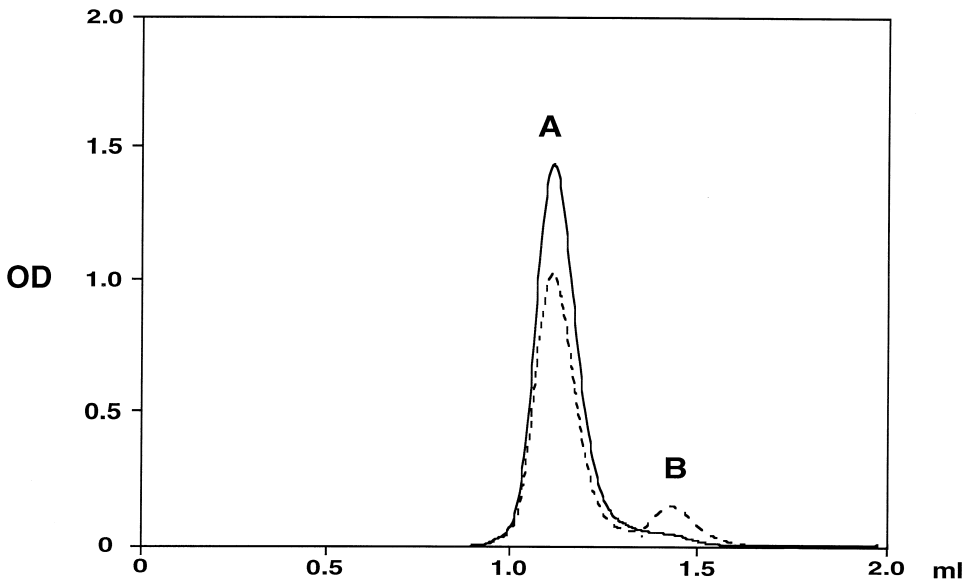

Fig. 1. Elution profile at 435 nm (solid line) and 598 nm (dotted line) of the purified PscA/PscC complex in 10 mM Tris, 0.1% NaN_3, 0.15% Triton X-100, pH = 8 containing 50 mM NaCl. Flow rate 0.1 ml/min on an gel filtration column Sephadex 200 3.2/30 attached to a SMART system. The protein concentration was 2 mg/ml and the sample volume 0.05 ml.

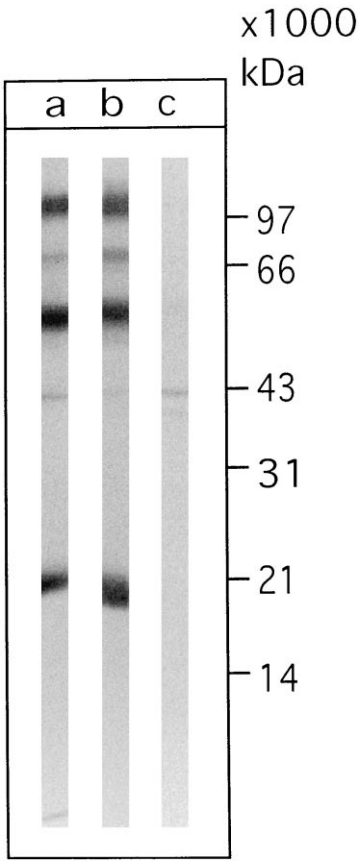

Fig. 2. Silver stained SDS–PAGE of the purified complex and the eluted fractions shown in Fig. 1. Isolated complex (lane a), fraction A (lane b) and fraction B (lane c).

gradient allowed fractionation of the extract. The brown RC containing fraction was used for structural analysis [10].

Gel filtration experiments were performed to obtain information about the oligomeric state of the isolated PscA/PscC complexes. In addition, different salt concentrations were used to study the interaction of the FMO with the PscA/PscC complexes, since the association of two forms of the FMO protein (trimer and monomer) has been proposed [37]. Fig. 1 shows the elution profiles recorded at 435 nm (solid line) and 598 nm (dotted line) of the RC complexes from *Chlorobium tepidum* in the presence of 50 mM NaCl in buffer A. The isolated PscA/PscC particles eluted as a main fraction (peak A) with an additional small protein fraction (peak B). The different absorption ratios of the two peaks at 435 and 598 nm indicated the presence of two different complexes rather than different aggregation states of the same complex. Similar elution profiles were obtained in the presence of 10, 150, 350 and 500 mM NaCl (data not shown) indicating that the presence of sodium chloride had no effect on the aggregation state of the purified complexes.

The protein composition of the isolated RC complexes is shown in Fig. 2, lane a. The SDS gels show protein bands at 100, 70, 55, 42 and 20 kDa. The diffuse band at 55 kDa is the PscA protein. This protein with a calculated molecular weight of 82 kDa [6] is known to produce a diffuse band at 55–60 kDa on SDS gels similar to the PsaA and PsaB proteins in PSI [9]. The low mass band at 20 kDa corresponds to the PscC subunit which was identified by heme-staining (data not shown). The relatively faint 42 kDa band is the BChl *a* carrying protein, FMO. Similar to

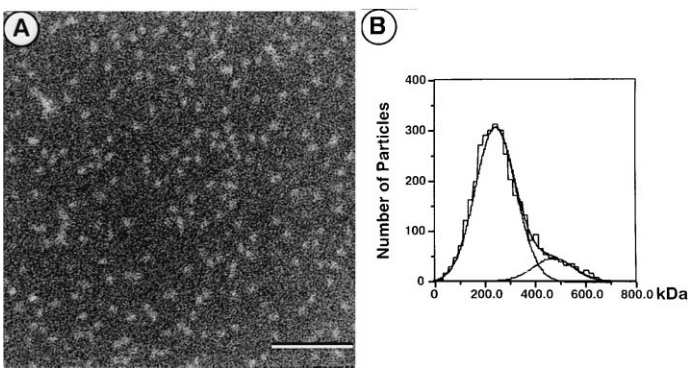

Fig. 3. Mass analysis of the purified PscA/PscC complex from *Chlorobium tepidum*. (A) STEM dark-field image of freeze-dried unstained PscA/PscC complexes. The scale bar is 100 nm. (B) Mass histogram of PscA/PscC particles ($n = 3557$). The Gauss peaks fitted at 231 ± 58 and 481 ± 61 kDa, respectively. The imaging dose was 332 ± 17 e/nm^2.

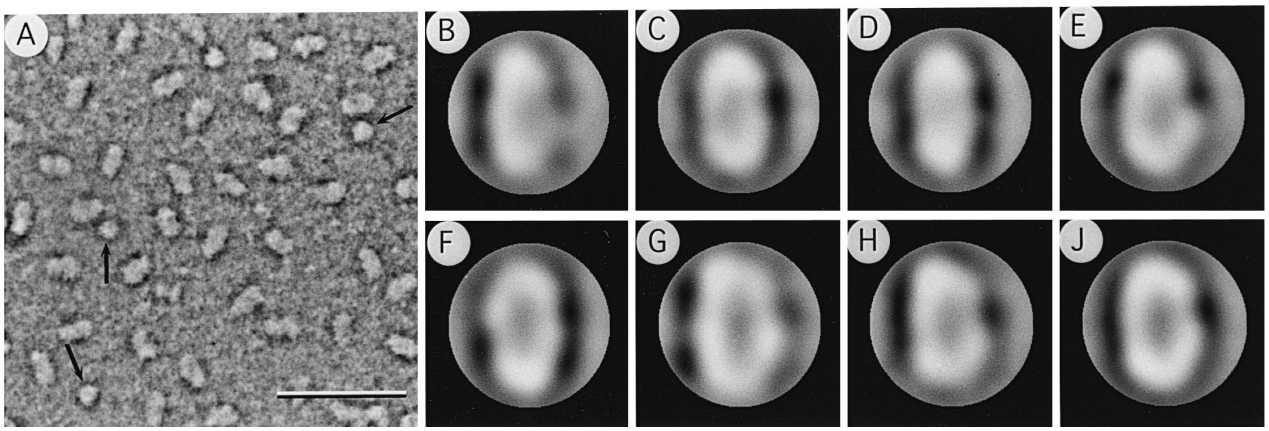

Fig. 4. Electron microscopy and classification of negatively stained PscA/PscC complexes. (A) STEM dark field electron micrographs of *Chlorobium tepidum* PscA/PscC complexes. The contrast has been adapted to show the protein in light shades. Arrows mark the small particles identified as FMO proteins. The scale bar represents 50 nm. (B–J) Classification of 1690 projections, which were aligned and treated with multivariate statistical analysis. The data set was decomposed into 8 classes with 152 (B), 93 (C), 146 (D), 128 (E), 261 (F), 318 (G), 147 (H), and 186 (J) members, respectively. The classified particles shown thus correspond to 85% of all particles.

PSI, aggregation of the subunits led to the two bands at 70 and 100 kDa whose appearance depended on the sample incubation temperature and the lapse time before loading the sample onto the SDS gels [26]. Fig. 2, lane b and c shows the SDS–PAGE from the pooled fractions. While the PscA, PscC proteins and trace amount of the FMO were present in peak A (lane b), peak B only contained the FMO protein (lane c). Similar protein compositions were obtained from the corresponding runs in the presence of 150, 350 and 500 mM NaCl (data not shown).

The homogeneity of the purified complexes was also assessed by analytical ultracentrifugation. In the presence of 50 mM NaCl, the purified RC complexes

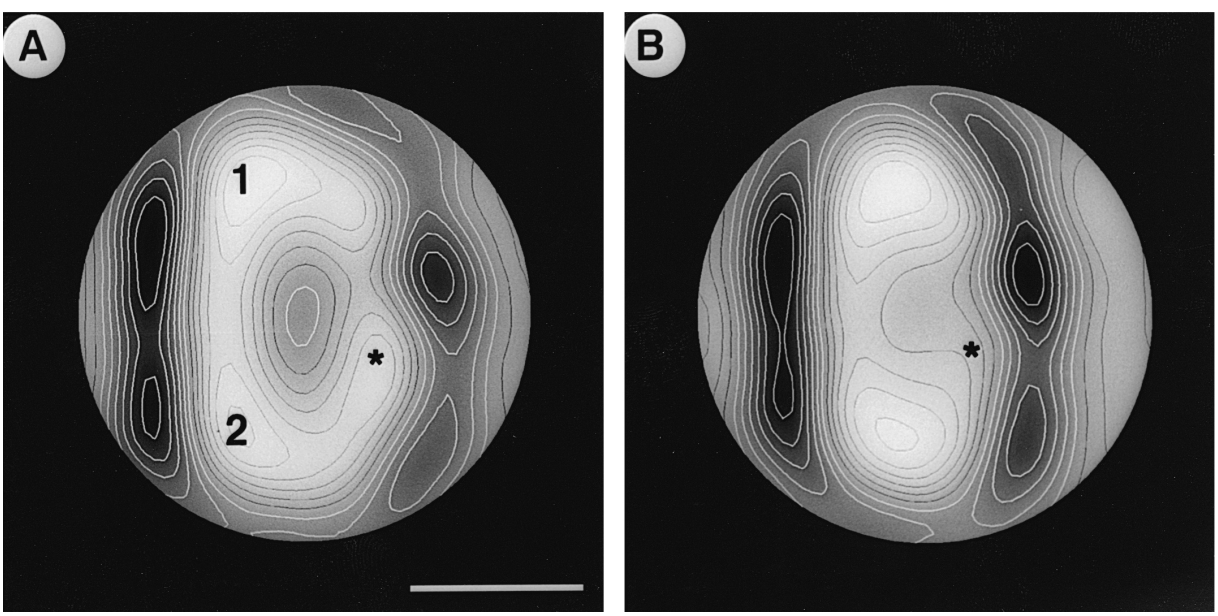

Fig. 5. Two views of the PscA/PscC complex. (A) Sum of 651 top view projections. (B) Sum of 391 side view projections. The scale bar represents 5 nm.

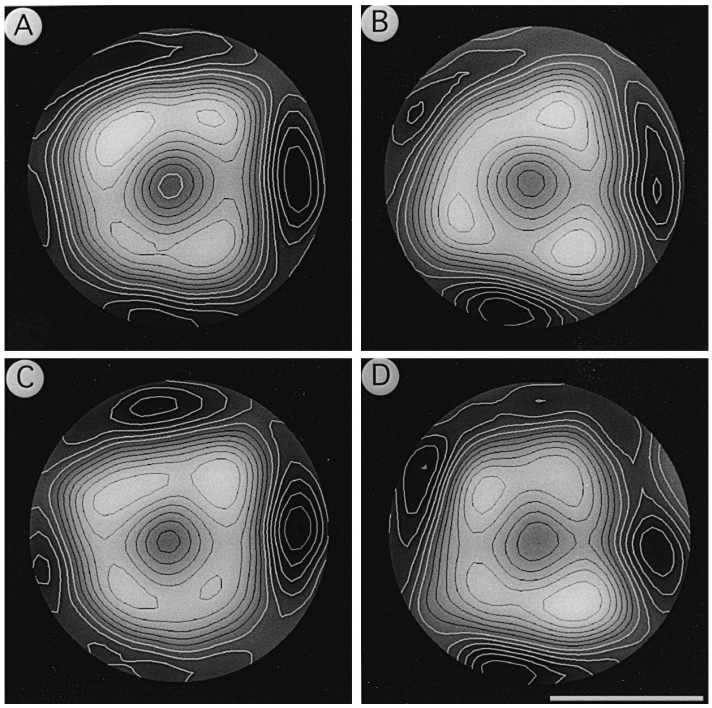

Fig. 6. Image analysis of 150 small particles. Top view projections were aligned, classified by multivariate statistical analysis, and averaged. Number of particles in averages A–D are 34, 26, 16 and 14, respectively, representing 78% of the selected particles. The scale bar represents 5 nm.

showed a main population with a sedimentation coefficient of 7.5 S. Sample homogeneity was unchanged by use of high salt concentrations. Thus, the data correlate to the gel filtration experiments, allowing the 7.5 S sedimentation coefficient to be assigned to the PscA/PscC complex.

3.2. Mass measurement of the RC complexes with the STEM

Mass analysis of freeze-dried unstained Triton X-100 solubilized RC complexes was performed with the STEM. The PscA/PscC complex was diluted (1 : 1000) with buffer A containing 10 mM NaCl prior to adsorption and washing for mass measurement. Complexes of two different sizes could be visually distinguished on the images (Fig. 3(A)), and were selected accordingly for the evaluation. The pooled mass data are shown in Fig. 3(B). Two Gaussian curves were fitted yielding peaks at 231 ± 58 kDa and 481 ± 58 kDa. Taking the imaging electron dose of $332 \pm 17\,e/nm^2$ ($n = 3557$) into account, these values become 242 ± 61 and 505 ± 61 after correction for beam induced mass loss, assuming the mass loss kinetics of the 500 kDa protein selenocysteine synthase [30]. A similar mass histogram (data not shown) yielding a main mass peak at 255 ± 60 kDa ($n = 4097$) after correction for mass loss was obtained from a sample diluted (1 : 1000) with buffer containing 500 mM NaCl. Comparison of the mass histograms, the SDS gels, and the elution profiles from gel filtration allow the mass of 248 kDa to be assigned to peak A complexes which only contained the PscA and PscC proteins. The relatively ill defined higher mass region ($\sim$ 510 kDa) probably arises from some higher aggregation states of these proteins.

3.3. Structural analysis of the PscA / PscC complexes by STEM

STEM images of negatively stained RC complexes recorded at 100 ekV were used for structural analysis (Fig. 4(A)). Well preserved particles ($n = 1690$) were aligned angularly and translationally, and submitted to a multivariate statistical analysis with subsequent classification. The projections were analyzed without imposing any symmetry. The classification separated the particles in eight major classes. Three classes

containing 24% of the data set are side views of the complex (Fig. 4(B–D)). Five classes containing 39% of the data set are various top views of the complex (Fig. 4 (E–J)). All top view classes show the same handedness. Small variations in the width of these class averages are probably the result of slight tilting of the molecules on the carbon support film. Averages of closely related top and side views were added to yield Fig. 5(A) (n = 651, sum of 4G, 4H, 4J) and 5B, respectively (n = 391, sum of 4(B–D)). From independent averages of the particles accumulated in the average displayed in Fig. 5(A), the resolution values determined by the ring correlation function [31], the phase residual [32] and the spectral signal-to-noise ratio [33] are 1.3, 1.8 and 1.8 nm, respectively. The top view projection (Fig. 5(A)) shows an elongated particle 13.0–14.0 nm long and 7.5–7.8 nm wide. Two peripheral domains are separated by a central cavity which is open on one side. The major protrusions (labeled as 1 and 2) are similar in shape giving the complex a pseudo two-fold symmetry. The putative side view projection (Fig. 5(B)) exhibits dimensions of 13.9 × 5.8 nm. Both the length of the particle and the separation of the two high protein densities are similar to those observed in the top view projection, but the central indentation is less pronounced.

The same electron micrographs also showed the presence of small particles in the sample (Fig. 4(A), arrows). Their projections (n = 150) were selected separately, aligned and decomposed into four main classes by multivariate statistical analysis and classification (Fig. 6). Visual inspection of the projections revealed the presence of triangular and almost square structures with a diameter of 8 nm. Three or four protein densities can be distinguished.

4. Discussion

Several solubilized, photoactive RC preparations from *Chlorobia* have been reported (for a review see [4]). The isolated complexes have been characterized by different spectroscopic methods such as absorption spectroscopy, EPR and FTIR. Based on these results, the green sulfur bacteria were proposed to contain electron acceptors similar to PSI [15,17,38–40]. This hypothesis has been confirmed by the sequence of the reaction center polypeptides from *Heliobacillus mobilis* and *Chlorobium limicola* f.sp. *thiosulfatophilium* [6,7]. Alignment of the PscA and PshA with the PsaA and PsaB sequences revealed the presence of a highly conserved dodecapeptide which contains two cysteins [6,7]. It has been suggested that these cysteins in the PSI dodecapeptide bind the F_X center between PsaA and PsaB [9]. A homodimeric core has thus been proposed for green sulfur bacteria based on the presence of a single gene for the reaction center core protein PscA and the existence of an Fe–S cluster similar to F_X [6,7]. Although 2D and 3D crystals of the PSI reaction center from *Synechoccus* sp., *Phormidium laminosum* and *Synechococcus* PCC 7002 have been produced [22–27], and the structure has been determined to 4 Å resolution [28,29], not much is known about the structure of the RC of green sulfur bacteria.

In the present work, we have studied an RC complex containing the PscA and PscC subunits from the green sulfur bacterium *Chlorobium tepidum* isolated by the method of Hager-Braun et al. (1995). HPLC gel filtration, SDS–PAGE and analytical ultracentrifugation indicated the presence of a homogenous complex, contaminated by spurious amounts of the FMO protein. The mass of 248 kDa determined by STEM for the solubilized complex is consistent with the presence of two copies of PscA (2 × 82.5 kDa) one copy of the PscC protein (20 kDa), 20 BChl *a* antenna molecules (20 × 1 kDa) and approximately 50 kDa assigned to detergent and or lipid. The latter mass is indeed close to that of a Triton X-100 micelle, 62–96 kDa, calculated from its aggregation number and molecular weight [41] or that of a lipid annulus containing about 70 lipid molecules. Based on spectroscopic data two PscC proteins per RC have been reported [19], a possibility that cannot strictly be excluded on the basis of the STEM mass data. The mass of 50 kDa assumed to be entirely related to detergent or residual lipids above would also be compatible with the presence of a second PscC in the RC. In conclusion, the STEM mass measurement presented here show the RC of the green sulfur bacteria to contain two copies of the PscA subunit and at least one copy of the PscC subunit.

Multivariate statistical analysis of STEM dark-field images from negatively stained specimens revealed a top and side view of the elongated particle (Fig. 5(A)

and (B), respectively). The height of the PscA/PscC complex, about 6 nm, agrees with that reported for monomeric cyanobacterial PSI complexes [42]. In addition, the length of the top views of the RC is close to that of the monomeric cyanobacterial complexes from two different strains [42,43]. Recently the structure of PSI from the cyanobacterium *Synechococcus* sp. at 4 Å resolution has been reported [29]. A comparison of the RC projection in Fig. 5(A) with the 4 Å structure of PSI reveals that they have similar length but different widths. The latter correlates with the absence of the small membrane proteins (PsaI–PsaM) of the RC in the green sulfur bacteria. The location of these small membrane proteins at the periphery of the PSI complex has been suggested [29]. The top and side view projections shown in Fig. 5 exhibit high protein densities at both ends of their long axes, and lower protein densities in the middle. The two major protrusions of the RC projection probably represent the large hydrophilic loops indicated by the predicted protein folding pattern of the PscA [6]. A distinct asymmetry of the top view indicated (asterisk in Fig. 5(A)) suggests that a single copy of PscC is present in the complex. The asymmetry of the side view suggests that the indentation is mainly on one side of the complex. Analogous to the cyanobacterial PSI complex which exhibits a pronounced indentation at the lumenal surface [27] the indentation observed with the RC is also proposed to be on the periplasmic side.

Gel filtration and SDS–PAGE (Figs. 1 and 2) show that the small particles observed in negatively stained preparations represent the FMO protein, which is supported by the small spherical particles occasionally found among the elongated RC particles (Fig. 4(A)). Image analysis of the small particles yielded triangular and square complexes (Fig. 6). The triangular particles observed (Fig. 6(B)) have a stain indentation about the three-fold axis and their size and shape are in agreement with the trimeric FMO structure [16]. Since PscD tends to associate with the FMO protein [10], the square complexes shown in Fig. 6(A), (C), (D) may reflect its presence.

Depending on the detergents used for their solubilization, both monomeric and trimeric forms of cyanobacterial PSI complexes have been isolated [22,26,42–44]. Recently, several groups have presented evidence for trimeric PSI complexes in thylakoid membranes [45–48]. The gel filtration experiments (Fig. 1) and the mass of 248 kDa determined for the PscA/PscC complex (Fig. 3) document the absence of a trimeric RC complex in the green sulfur bacterium *Chlorobium tepidum*. This may be explained by the absence of a homologous protein to the PsaL protein of cyanobacterial PSI complexes, which is essential for trimerization of the PSI complex [49].

Based on crystallographic data, a pseudo two-fold symmetry axis has been proposed for the photosynthetic Q-type RCs and the Fe–S RCs [29,50]. Recently, the existence of such a pseudo two-fold symmetry axis was also reported for the monomeric form of the PSII complex, a Q-type RC [51–55]. The existence of two copies of PscA in the reaction center in the green sulfur bacteria supports the idea of a RC complex with a pseudo two-fold symmetry, in excellent agreement with the top view and side view projections of the negatively stained complexes.

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