Isolation and Properties of Photochemically Active Reaction Center Complexes from the Green Sulfur Bacterium *Prosthecochloris aestuarii*[†]

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ABSTRACT: A new and rapid procedure was developed for the isolation of the reaction center core (RCC)-complex from the green sulfur bacterium *Prosthecochloris aestuarii*. Reaction center preparations containing the Fenna Matthews Olson (FMO) protein were also obtained. The procedure involved incubation of broken cells with the detergents Triton X-100 and SB12, sucrose gradient centrifugation and hydroxyapatite chromatography. Three different pigment protein complexes were obtained: one containing (about) three FMO trimers per RCC, one with one FMO per RCC and one consisting of RCC only. The last one contained polypeptides with apparent molecular masses of 64 kDa (pscA) and 35 kDa (pscB, the F_A/F_B, FeS subunit), but no cytochrome. Bacteriochlorophyll *a* and the chlorophyll *a* isomer functioning as primary electron acceptor were present at a ratio of 4.8:1. The complexes were also characterized spectroscopically and in terms of photochemical activity, at room temperature as well as at cryogenic temperatures. Illumination caused oxidation of the primary donor P840, with the highest activity in the RCC complex ($\Delta A_{840}/A_{810} = 0.06$). At room temperature in the RCC complex essentially all of the $P840^+$ produced in a flash was re-reduced slowly in the dark (several seconds). At low temperatures (150−10 K) a triplet was formed in a fraction of the reaction centers, presumably by a reversal of the charge separation, whereas in others $P840^+$ formed in the light was re-reduced in 40−50 ms.

The pigment system of green sulfur bacteria consists of three components: the chlorosome, the FMO protein, and the reaction center core (RCC) complex. The absorption spectrum of intact cells is dominated by the chlorosomes, which contain bacteriochlorophyll (BChl) *c* or the related pigments BChl *d* and *e*, and have Q_Y absorption maxima in the region 720−750 nm (*1*). The FMO protein is organized in trimers. It contains 7 BChls *a* per monomer and is assumed to be situated between the chlorosome and the RCC complex. The RCC complex is embedded in the cytoplasmic membrane (*1,2*). The FMO protein as well as the RCC complex absorbs in the region 790−850 nm.

An isolation procedure for membranes free of chlorosomes was first described by Fowler et al. (*3*). Such membranes were designated "Complex I" (*4*) and contained both the RCC complex and the FMO protein. Most of the FMO protein could be removed with the aid of chaotropic agents and the resulting preparation was called "Complex II" (*4*). Detergent solubilization of Complex I by Swarthoff and Amesz (*5*) yielded a complex which was called "photosystem protein (PP) complex", and the detergent-solubilized equivalent of Complex II was named the "reaction center pigment protein (RCPP) complex". Removal of the residual FMO protein led to a complex which was called the "core complex" (*6*). However, this core complex was photochemically inactive.

Various other methods have been developed for the isolation of "reaction center complexes" from green sulfur bacteria (for reviews see refs 2 and 7). Most preparations contained four or five major polypeptides: the reaction center protein (the *pscA* gene product) with an apparent molecular mass of ca. 65 kDa, a protein carrying the FeS centers F_A and F_B (*pscB*, ca. 32 kDa), a cytochrome *c* (20−30 kDa), the *pscD* gene product, of unknown function (*8*) (17 kDa), and the FMO protein (42 kDa). The absorption spectra of the isolated complexes were very similar to either that of the PP complex (*8*−*10*), the RCPP-complex (*10*−*13*), or the core complex (*14*−*16*); characteristic is the ratio of the absorbances at 810 nm and at the shoulder at 835 nm (A_{810}/A_{835} ratio). This ratio is about 4.0−4.4 for the first type (*5, 9, 10*) and 2.2−2.8 for the second type of preparation (*5, 10, 13, 17*), whereas for the core complexes an A_{810}/A_{835} ratio of 1.3−1.7 has been reported (*2, 6, 14, 16*). The corresponding molecular masses were 600 kDa (*5*), 330−350 kDa (*5, 10*) and about 200 kDa (*6*), respectively. The number of BChls *a* per reaction center was estimated at 70−100 (*8, 10*), 35−40 (*5, 10, 13*) and 15−25 (*6, 14, 16, 18*), respectively. These data indicate that the PP and related complexes contain about three FMO protein trimers and that the RCPP and related complexes contain one FMO protein trimer or dimer. Hence, we will denote these complexes as the 3FMO−RCC and the 1FMO−RCC complexes throughout this paper.

In contrast to reaction centers of purple bacteria, which are devoid of antenna pigment, the RCC complex of green sulfur bacteria contains a considerable amount of antenna

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[1] Abbreviations: BChl, bacteriochlorophyll; Chl, chlorophyll; FMO-protein, Fenna Matthews Olson protein; HPLC, high-performance liquid chromatography; P840, primary electron donor; PMS, *N*-methylphenazonium methosulfate; RCC, reaction center core; SB12, sulfobetaine-12; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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BChl *a*. However, the number of pigment molecules bound to the RCC complex is not well established. Besides 15−25 BChls *a*, between 3 and 13 molecules of a chlorophyll (Chl) *a* isomer and 3−11 carotenoid molecules are reported to be bound to the reaction center protein (*16*, *18*, *19*). Two of the BChl *a* molecules constitute the primary electron donor P840 (*20*−*22*), while the isomer of Chl *a* (*19*, *23*), absorbing near 670 nm, appears to function as primary electron acceptor (*24*).

Up to now, methods for the preparation of "reaction center complexes" from green sulfur bacteria involved the breaking of cells, the fractionation of the cell fragments (chlorosomes and membranes), detergent solubilization of the resulting cytoplasmic membranes, and purification of the solubilized membranes by means of centrifugation and/or chromatography. These methods are not only laborious, but the photochemical activity of, in particular, the RCC preparations thus obtained was not very satisfactory (*2*, *6*, *16*). This paper describes a new and rapid procedure for the isolation of a photochemically active RCC complex and two different FMO−RCC complexes from *Prosthecochloris aestuarii*. The preparations were characterized by their absorption spectra, and their pigment and subunit composition were analyzed by means of HPLC and SDS−PAGE, respectively. The RCC complex showed a high photochemical activity, at room as well as at low temperatures.

MATERIALS AND METHODS

P. aestuarii strain 2K was grown as a mixed culture at a light intensity of 1 klux from incandescent lamps in the medium described by Holt et al. (*25*). Cells were harvested by centrifugation and resuspended in a buffer containing 50 mM Tris/HCl and 10 mM sodium ascorbate (pH = 8.3). They were then broken by sonication. The resulting suspension was centrifuged for 10 min at about 15000*g* and the supernatant was incubated for 3 h at 4 °C with 50 mM Triton X-100 and 25 mM SB12. Then the suspension was centrifuged at about 200000*g* for 1 h. The resulting supernatant contained the solubilized 3FMO−RCC complex while the pellet consisted of chlorosome material. Purification of the complex was achieved by sucrose gradient centrifugation [16 h at 300000*g*; 20−70% (w/v) sucrose, 50 mM Tris HCl (pH = 8.3), 10 mM sodium ascorbate and 2.5 mM Triton X-100]. The 3FMO−RCC complex settled at about 45% sucrose.

For the preparation of the RCC and 1FMO−RCC complexes the FMO protein was removed by hydroxyapatite chromatography. The purified 3FMO−RCC complex was loaded onto a hydroxyapatite column (Bio-Rad, DNA Grade Bio-Gel HTP) equilibrated with a buffer containing 20 mM potassium phosphate (pH = 6.5), 10 mM sodium ascorbate and 2.5 mM Triton X-100. Most of the sucrose was first removed by diluting the preparation 10-fold with the equilibration buffer and subsequent concentration over a 100 kDa membrane filter (Pall Filtron). The column was washed with the same buffer, containing 500 mM LiCl; the eluate contained the RCC complex. Elution with a buffer of pH 7.0, without LiCl, yielded the 1FMO−RCC complex. All procedures were performed in dim light and at 4 °C, except for the chromatography step which was performed at room temperature.

SDS−PAGE was performed as described by van de Meent et al. (*26*), except that the sample was not heated and no urea was used. The gels were stained with Coomassie Brilliant Blue. For pigment analysis, the preparations were extracted with a mixture of methanol and acetone (1/1, v/v) as described elsewhere (*27*). The extracts were analyzed by reversed phase HPLC on a C18 silica column (Chrompack, Spherisorb 5 ODS2, 250 × 4.6 mm i.d.) using a mixture of methanol and acetone (9/1, v/v) as eluent. Pigment elution was monitored by means of a Jasco MD-915 diode array detector.

Room and low temperature steady state absorption spectroscopy was performed using a single-beam spectrophotometer with a spectral resolution of 0.5 nm. Absorbance difference spectra induced by continuous illumination were measured with an apparatus described by Visser (*28*). Flash-induced absorption changes were measured as described by Franken and Amesz (*29*). Excitation flashes were provided by a Q-switched frequency-doubled Nd:YAG laser (15 ns, 532 nm, 10 Hz, 90 mJ per pulse). Measurements at cryogenic temperatures were performed using a helium flow cryostat (Oxford Instruments or Ultreks-LSO, Estonia). To obtain clear samples at low temperature glycerol (66% v/v) was added.

RESULTS AND INTERPRETATION

Absorption Spectra. The absorption spectra of the 3FMO−RCC and 1FMO−RCC complexes, measured at room and at low temperature, are given in Figure 1, A and B. In the Q_Y and Q_X region (550−850 nm), the spectra resembled those of the PP and RCPP complexes (*5*, *30*), respectively. However, the absorbance in the region 400−550 nm indicated that the carotenoid content was considerably lower and comparable to that of other "reaction center preparations" (*8*, *10*, *12*, *13*). At room temperature the Q_Y absorption maxima were located at 810 and 812 nm and the A_{810}/A_{835} ratios were 5.5 and 2.8, respectively. The first number is rather high compared to the ratios earlier obtained for similar preparations (see introduction), what might indicate that there were more than three FMO protein subunits present per RC. The second derivatives of the low-temperature spectra of both FMO−RCC preparations (not shown) displayed Q_Y absorption bands located at 801, 805, 815, 826, and 837 nm. The band at 837 nm was relatively strong in the derivative spectrum of the 1FMO−RCC complex, in agreement with the absorption spectrum.

Figure 1C shows the low temperature absorption spectrum of the RCC complex. The band at 837 nm was considerably higher compared to the other bands in the Q_Y region than in the FMO−RCC complexes, and the same was true for the band near 668 nm, due to the Chl *a* isomer mentioned earlier. The positions of the absorption bands in the region 790−840 nm were determined from the second-derivative spectrum; they were located at 795, 800, 809, 819, 830, and 836 nm. The band at 826 nm, which is typical for the FMO protein (*1*), was lacking. The room temperature absorption spectrum of the RCC complex (Figure 1C, inset) was nearly identical to those reported by Oh-oka et al. (*16*) and Hauska and co-workers (*2*, *14*), with an A_{810}/A_{835} ratio of 1.6. The low-temperature spectrum also resembles those of the other RCC complexes (*6*, *14*, *16*); the bands are located at approximately the same position as in the "calculated" spectrum of ref 31, but their relative amplitudes are different.

Protein and Pigment Composition. The protein composition of the three preparations was determined by means of

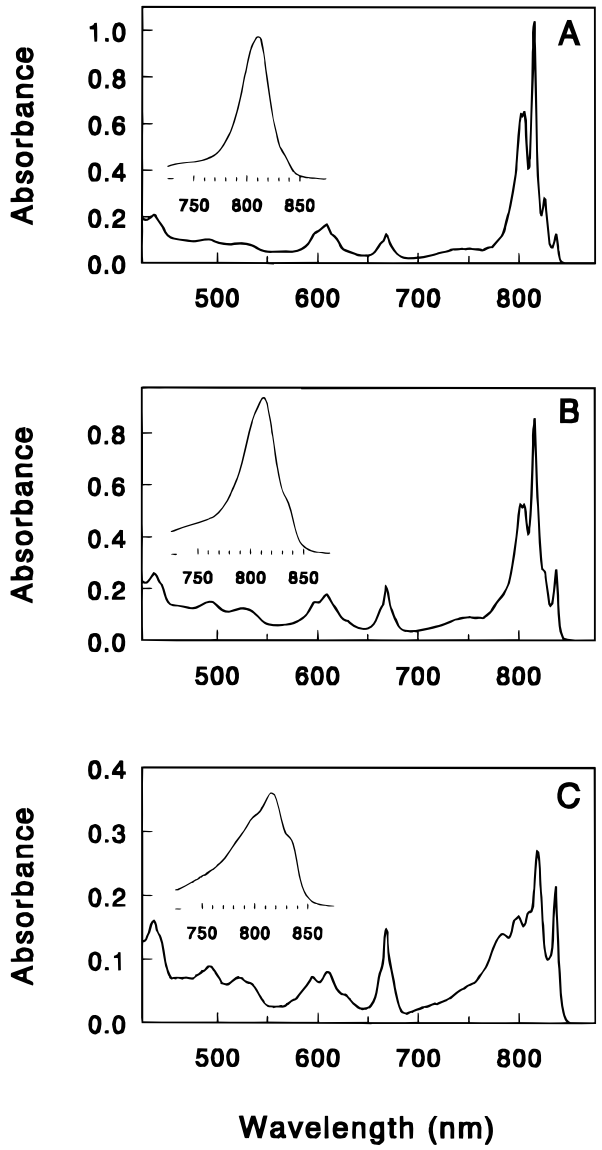

FIGURE 1: Absorption spectra of the (A) 3FMO−RCC, (B) 1FMO−RCC, and (C) RCC complex measured at 6 K. The Q_Y regions of the spectra measured at room temperature are shown in the insets.

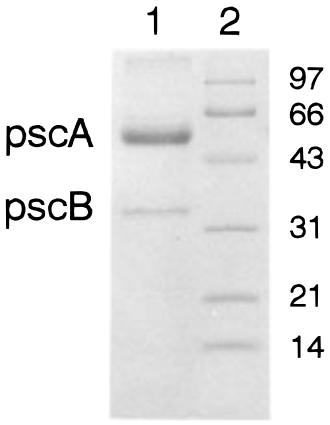

FIGURE 2: SDS−PAGE of the RCC complex (lane 1). Lane 2 contains molecular weight markers.

SDS−PAGE. The RCC complex gave two prominent bands corresponding to apparent molecular masses of 64 and 35 kDa together with a minor band of 125 kDa (Figure 2). The 64 kDa band belongs to the reaction center protein (*pscA*); the 125 kDa band is obviously due to its dimer. The 35 kDa band probably corresponds to the 32 kDa band that was observed by Vasmel et al. (*6*) and Hurt and Hauska (*14*) and presumably represents the protein carrying the FeS centers F_A and F_B (*pscB*, see ref 2). The 20 kDa cytochrome *c* (*pscC*), present in the RCC complexes from *Chlorobium limicola* f. *thiosulfatophilum* (*14*, *15*), was not present in our preparation and no heme-staining bands were observed. No evidence was found for the presence of either FMO (42 kDa) or *pscD* protein (17 kDa).

Just like the PP and RCPP complexes isolated by Swarthoff and Amesz (*5*), our 3FMO−RCC and 1FMO−RCC preparations contained many protein components; the preparations showed major bands of about 65, 47, 40, and 35 kDa and many additional small bands. The 40 kDa band may be ascribed to the FMO protein. Pigment analysis was performed by reversed phase HPLC. The main pigment was BChl *a* in all cases, esterified with phytol, but with minor amounts (about 1%) that were esterified with its metabolic precursors (*27*), eluting before BChl *a*. A smaller peak, eluting somewhat more slowly, was due to the Chl *a*-isomer. This pigment was clearly enriched in the RCC complex; using a molar extinction coefficient of 46 mM^{-1} cm^{-1} at 771 nm for BChl *a* (32) and of 77 mM^{-1} cm^{-1} at 665 nm for the Chl *a* isomer (33), we arrived at a molar ratio BChl *a*: Chl *a* isomer of 11.4 for the 3FMO−RCC complex and of 4.3 for the RCC complex. Both preparations showed two major carotenoid bands, probably due to rhodopin and to chlorobactene, respectively (*34*, *35*), as judged from their absorption spectra and retention times. Small amounts of BChls *c*, eluting before BChl *a*, were found in the 3FMO−RCC complex. The extracts from both complexes showed in addition a minor peak, which, on basis of its absorption spectrum, could be assigned to bacteriopheophytin *a*. This pigment is probably an artifact, produced during the isolation procedure. If we assume that it is derived from BChl *a*, then the "true" ratio of BChl *a* to Chl *a* isomer for the RCC complex would be 4.8 and for the 3FMO−RCC complex 11.8.

Photochemical Activity. The activity of the preparations was checked by measuring the light-induced bleaching at 840 nm. PMS and ascorbate were added to keep P840 reduced in the dark. With saturating continuous illumination, the bleaching at 840 nm divided by the absorbance at 810 nm ($\Delta A_{840}/A_{810}$) was 0.012 for the 3FMO−RCC complex, comparable to the numbers found for isolated membranes (*3*) and for the PP complex (*5*), respectively. The light-induced bleaching of the 1FMO−RCC complex ($\Delta A_{840}/A_{810} = 0.020$) was comparable to the chemically induced absorbance difference found by Okkels et al. (*11*) and Oh-oka et al. (*12*) but was about half as large as the light-induced signal observed by Swarthoff and Amesz (*5*) in the RCCP complex. A quite high activity was observed with the RCC complex, with $\Delta A_{840}/A_{810}$ ranging from 0.056 to 0.063 for different preparations. This number is about 70% higher than that observed by Oh-oka et al. (*16*) upon chemical oxidation of their RCC preparation and about 5 times higher than observed for the 3FMO−RCC complex. (Since the absorption maximum of the RCC complex is at 815 nm, it may be more appropriate to compare $\Delta A_{840}/A_{max}$ for both preparations, which are 0.012 and 0.052−0.058, respectively). The absorption difference spectrum of the RCC complex (not shown) was very similar to those earlier observed with the PP and RCPP complexes (*5*).

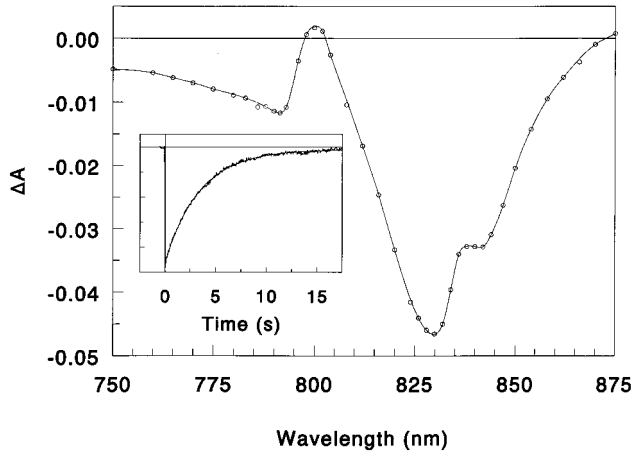

FIGURE 3: Difference spectrum and kinetics at 830 nm (inset) of the absorbance changes induced by a laser flash at 532 nm in the RCC complex at 10 °C. Conditions: 20 mM potassium phosphate (pH = 6.5), 50 mM LiCl, 10 mM sodium ascorbate, 2.5 mM Triton X-100, 50 μM PMS. Each point was obtained as the average of 2−4 recordings, obtained at 25 s intervals. The smooth line in the inset represents a monoexponential decay with a time constant of 4.0 s.

Kinetics of flash-induced absorbance changes are given in Figure 3 (inset). A rapid bleaching occurred, which was completed within the time resolution of our apparatus and was followed by a slow exponential decay with a time constant of 4 s. Thus, the $P840^+$ formed in a flash appeared to be quite stable. Figure 3 also shows the corresponding absorption difference spectrum, which was identical to that obtained by continuous illumination. In contrast to similar measurements with isolated membranes (*29*), essentially no triplet formation was observed at room temperature. A minor decay component of 70 μs was observed, but its amplitude was too small to obtain a reliable difference spectrum. A 70 μs component was also seen by Miller et al. (*36*) in a membrane preparation of *Chlorobium limicola* and ascribed to a recombination of $P840^+$ with the reduced first iron sulfur acceptor F_X^-.

The RCC complex was rather unstable. When stored in the dark at 25 °C for 2 h, the absorption spectrum changed, with the Q_Y maximum shifting from 815 to 785 nm, and the photochemical activity was strongly reduced. When rapidly frozen and stored under liquid nitrogen, however, the preparation was quite stable and upon rewarming the activity and the absorption spectrum had not changed significantly. All experiments, except for the activity measurements described above, were done with such a preparation.

Low-Temperature Photochemistry. As shown in Figure 4A,B, biphasic decay kinetics were observed at low temperature. Figure 4C shows the temperature dependence of the two time constants involved. The time constant of the "slow" component rapidly decreased upon cooling and reached a value of 40−50 ms below 150 K, while that of the rapid component increased from 200 μs at 200 K to 350 μs at 10 K. The relative amplitudes of the two components were approximately constant at 1.2:1 over this temperature range.

Absorption difference spectra of the two components at 10 and 100 K are shown in Figure 5. The spectra of the slow component (Figure 5A and B) resemble that obtained with the PP complex at 80 K (*37*), but they show more detail, with minima at 838, 834, 824, 815, 805, and 793 and maxima

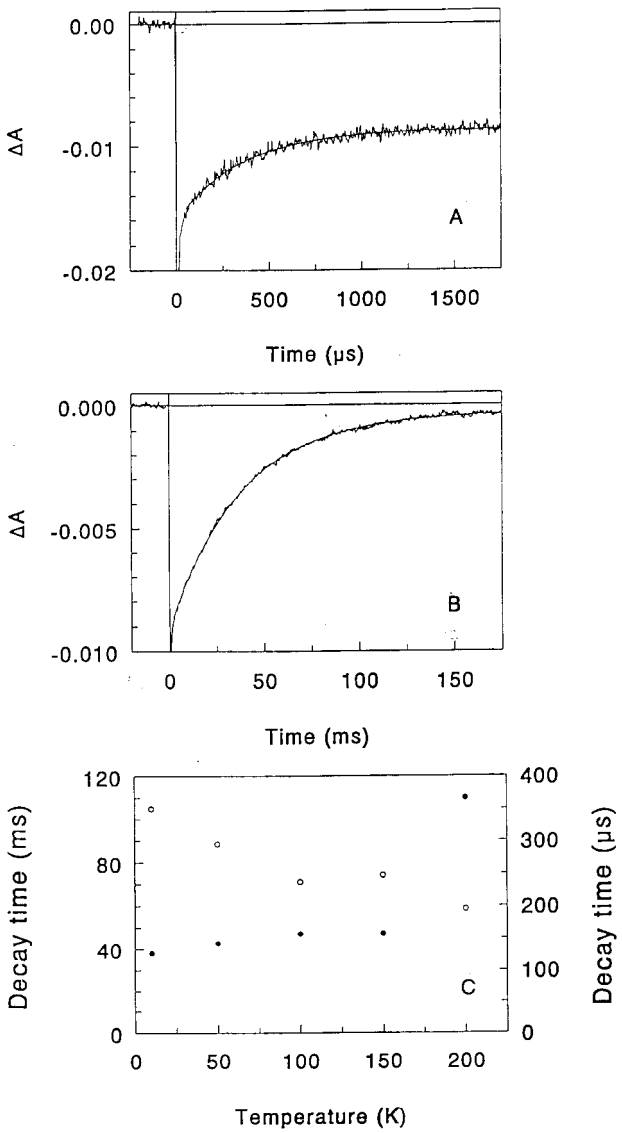

FIGURE 4: (A, B) Kinetics of flash-induced absorbance changes at 837 nm of the RCC complex at 10 K. Each trace is the average of 50 (A) or 30 (B) recordings, obtained at 1.5 s intervals. A biphasic decay is observed after the initial bleaching. Recording B was obtained with a low-pass filter to cutoff frequencies above 1 kHz. The smooth lines are monoexponential fits with time constants of 350 μs (A) and 38 ms (B), respectively. (C) Time constants of the rapid (open circles, right-hand scale) and of the slow decay components as a function of temperature. Conditions as for Figure 3, except glycerol 66% (v/v), 6 mM potassium phosphate, 16 mM LiCl, and 1 mM Triton X-100 were used.

at 835, 830, 818, 812, and 800 nm (10 K). These spectra can be assigned to photo-oxidized P840. A difference spectrum of the rapid component is shown in Figure 5C. With a main bleaching at 837 nm, the spectrum is probably due to the core reaction center BChl *a* triplet that has a lifetime of 165 μs in isolated membranes at room temperature (*29*). The second minimum, at 825 nm, was not resolved at room temperature, but a similar minimum was observed by Vasmel et al. (*38*) at 1.2 K by absorption detected magnetic resonance.

DISCUSSION

Our results show that it is possible to obtain reaction center core complexes from green sulfur bacteria, as well as RCC complexes associated with varying amounts of the FMO

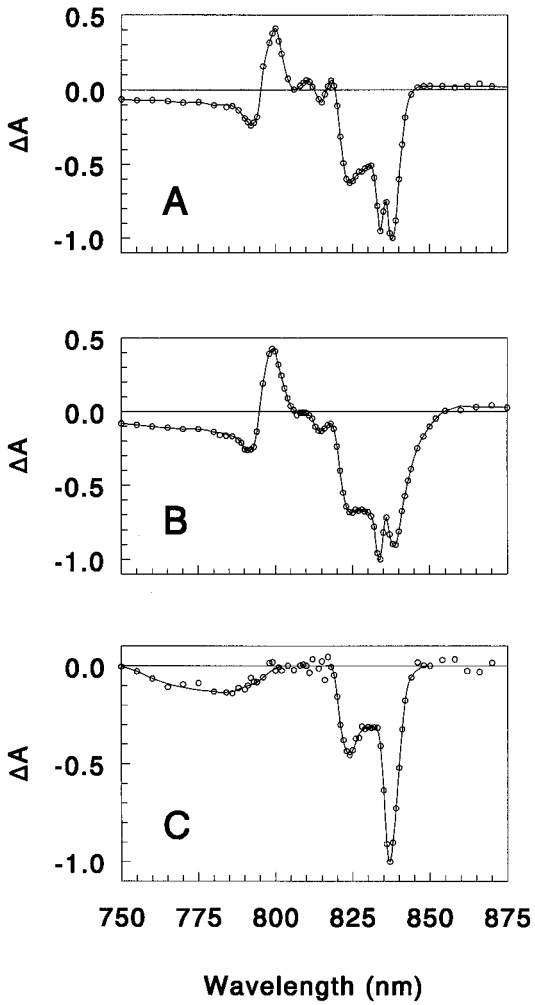

FIGURE 5: Absorption difference spectra at low temperatures. (A, B) Spectra of the slow decay component (40−50 ms) at 10 and 100 K, respectively. (C) Spectrum of the 350 μs component at 10 K. Conditions as for Figure 4.

protein complex by means of relatively fast and simple procedures. The main difference with other methods is that we applied detergent solubilization of sonicated cells, rather than of isolated cytoplasmic membranes. Preliminary experiments indicate that the same procedure can be also applied to other species of green sulfur bacteria (*Cb. phaeovibrioides*, *Cb. tepidum*, and *Cb. vibrioforme*).

The RCC complex in particular shows a high photochemical activity, with a maximum bleaching at 840 nm which is 6.0% of the absorbance at 810 nm. Assuming the same extinction coefficients for P840 oxidation and for BChl *a in vivo* (34) and taking into account the results obtained by HPLC, we would arrive at a number of 17 BChls *a* and 3−4 molecules of Chl *a* isomer per reaction center.

The oxidized primary electron donor, $P840^+$ was remarkably stable in our RCC preparation and appeared to react only quite slowly with PMS and ascorbate. This is in contrast to the RCC complex of heliobacteria, where re-reduction of the oxidized donor took only a few tens of ms under similar conditions (*26*). There is no agreement on the rate of the back reaction between $P840^+$ and the reduced terminal electron acceptor, F_A- or F_B-, with time constants of 7 and about 500 ms being mentioned in the literature (*2, 7, 36, 39*). Nevertheless, the high stability of photo-oxidized P840 in our preparation suggests the absence of such a back reaction and indicates that the electrons are rapidly taken up by the medium. Whether this electron transfer occurs via the F_A/F_B subunit is not clear at present; it is possible that the F_A and F_B iron sulfur centers are destroyed by the treatment with chaotropic agent needed to remove the FMO protein (*36*).

Whereas little or no triplet formation occurred at room temperature, a BChl *a* triplet with a lifetime of a few hundred μs was formed in about half the reaction centers at low temperatures. This triplet, presumably located on P840 (*20, 38*) or on a nearby BChl *a* molecule, is probably generated by a reversal of the primary charge separation. In other reaction centers $P840^+$ was re-reduced in 40−50 ms at temperatures below 150 K, presumably by a back reaction with the reduced iron sulfur center F_{X^-} (*36*). This indicates that electron transfer to F_A and F_B, if it occurs at room temperature, is blocked below 150 K. A 20 times faster back reaction was observed in the RCC complex of heliobacteria at 5 K (*26*).

The low-temperature absorption spectrum of the RCC complex is different from that of the related complexes, the core of photosystem I and the RCC complex of heliobacteria, in showing a large number of well-resolved Q_Y transitions at low temperature, and, although the structure is quite different, in this respect it shows a remarkable similarity to that of the FMO protein. The main cause of this difference with respect to related photosystems may be a relatively low number of BChls *a* per reaction center, so that there is less overlap between the individual transitions. However, this does not explain the differences in the spectra of the oxidation of the primary electron donor. Whereas the photooxidation of P798 of heliobacteria shows a smooth difference spectrum with a single minimum at 793 nm at low temperatures (*40*), the difference spectrum of P840 oxidation is extremely complicated, showing many maxima and minima in the Q_Y region of BChl *a*, presumably caused by Stark effects on antenna BChl *a* molecules.

At this point it is of interest to compare the properties of our RCC complex, from *P. aestuarii*, with those prepared by others from *Cb. limicola* f. *thiosulfatophilum* (*14*−*16*). As mentioned already, the room temperature absorption spectra are similar, but in other respects there are clear differences. The RCC complex isolated by us consists of a 64 kDa core peptide and a 35 kDa protein, presumably the F_A/F_B subunit (*pscB*), but contains no *pscC* protein (cytochrome *c*). In contrast, the preparation of Oh-oka and co-workers (*16*) contained the *pscA* core peptide and cytochrome c_{551} (20 kDa), whereas that of Hauska and co-workers (*2*, *14*, *15*) contained in addition the F_A/F_B subunit, which was not present in the preparation of Oh-oka et al.

Oh-oka et al. (*16*) have published some details about the photochemical properties of their RCC complex. Apparently, no photochemical activity was observed at room temperature, but at 77 K biphasic kinetics were observed after a flash, with time constants of 30 and 150 μs. Both components were attributed to reaction center triplets, and in fact the difference spectra showed some resemblance to that of Figure 5C, although the signal-to-noise level does not permit a close comparison. Apparently, no $P840^+$ was stably formed in this preparation. The spectrum obtained upon 30 min illumination at 77 K (*16*) does not resemble the $P840^+$ difference spectrum (Figure 5B) and is more probably due to a structural change in the protein as observed under similar conditions in the FMO protein (*41*).

In summary we conclude that a relatively fast and simple method is now available for the preparation of a highly active RCC complex from green sulfur bacteria. Further studies of such complexes by optical and ESR methods may serve to elucidate the properties of the reaction center of these bacteria.

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