Dynamics of Energy Conversion in Reaction Center Core Complexes of the Green Sulfur Bacterium *Prosthecochloris aestuarii* at Low Temperature†

Sieglinde Neerken,* Kristiane A. Schmidt, Thijs J. Aartsma, and Jan Amesz

Department of Biophysics, Huygens Laboratory, Leiden University, P.O. Box 9504, 2300 RA Leiden, The Netherlands

Received May 13, 1999; Revised Manuscript Received July 26, 1999

ABSTRACT: Excited-state and electron-transfer dynamics at cryogenic temperature in reaction center core (RCC) complexes of the photosynthetic green sulfur bacterium *Prosthecochloris aestuarii* were studied by means of time-resolved absorption spectroscopy, using selective excitaton of bacteriochlorophyll (BChl) *a* and of chlorophyll (Chl) *a* 670. The results indicate that the BChls *a* of the RCC complex form an excitonically coupled system. Relaxation of the excitation energy within the ensemble of BChl *a* molecules occurred within 2 ps. A time constant of about 25 ps was ascribed to charge separation. Absorption changes in the 670 nm region, where Chl *a* 670 absorbs, were fairly complicated. They showed various time constants and were dependent on the wavelength of excitation and they did not lead to a simple picture of the electron acceptor reaction. Energy transfer from Chl *a* 670 to BChl *a* occurred with a time constant of 1.5 ps. However, upon excitation of Chl *a* 670 the amount of oxidized primary electron donor, $P840^+$, formed relative to that of excited BChl *a* was considerably larger than upon direct excitation of BChl *a*. This indicates the existence of an alternative pathway for charge separation which does not involve excited BChl *a*.

It is generally assumed that the reaction center of green sulfur bacteria resembles that of photosystem I of green plants (*1*). However, for studies of energy relaxation and electron transfer, the reaction center core (RCC)[1] complex of green sulfur bacteria offers at least two advantages over the corresponding photosystem I core complexes. First of all, the energy levels of the system involved in energy transfer are much better resolved, while the complex contains fewer pigments. Second, the putative electron acceptor, a chlorophyll *a* isomer, Chl *a* 670, absorbs in a spectral range far away from that of the "antenna" bacteriochlorophylls (BChls) and the primary electron donor.

The RCC complex of *Prosthecochloris aestuarii* presumably contains a total of 16 bacteriochlorophyll (BChl) *a* molecules and 4 Chls *a* 670 (*2*). The primary electron donor P840, a dimer of BChl *a*, is assumed to absorb at the low energy side of the BChl *a* Q_y band (near 837 nm at low temperature). The residual 14 BChls *a* form the core antenna, which absorbs between 780 and 840 nm. At least some of the Chl *a* 670 molecules are closely associated with the reaction center and may function as primary electron acceptors (*1*, 3−5). Perhaps the four pigments are arranged in a similar fashion as are the corresponding Chls in photosystem I (*6*).

For several years studies have been performed on the energy- and electron-transfer dynamics in membranes of green sulfur bacteria by means of transient absorption in the picosecond time range. Such membranes contain the Fenna−Matthews−Olson (FMO) antenna complex in addition to the core complex. These experiments were done with nonselective excitation at 532 nm and at room as well as at cryogenic temperature (*3*, *7*, *8*). Similar studies were also performed with FMO-containing preparations that were solubilized with detergents (*1*, *9*). Now that a fast procedure for isolating photoactive RCC complexes from green sulfur bacteria has been developed (*10*), more detailed studies of the dynamics of energy transformation have become possible. Recently we performed time-resolved measurements with selective excitation of the BChls *a* and Chls *a* 670 at 275 K (*11*). This paper reports the results of absorbance difference measurements at cryogenic temperatures. The experiments indicate that the RCC complex can be described in terms of an excitonically coupled system. Relaxation of the excitation energy within the ensemble of BChl *a* pigments occurred within 2 ps, and a time constant of about 25 ps is ascribed to charge separation. Selective excitation of Chl *a* 670 resulted in the formation of a larger amount of $P840^+$ in relation to that of excited BChl *a* than upon excitation of BChl *a*, indicating the existence of an alternative pathway for charge separation which does not involve excited BChl *a*.

MATERIALS AND METHODS

RCC complexes from the green sulfur bacterium *Prosthecochloris aestuarii* were prepared essentially according to Francke et al. (*10*) and dissolved in a buffer containing 20

† This work was supported by the Section for Earth and Life Sciences (ALW) and by the European Union (Contract No. FMRX-CT 96-0081).

* Author for correspondence. E-mail: sigi@biophys.leidenuniv.nl. Fax: +31 71 5275819.

[1] Abbreviations: A_0, primary electron acceptor; BChl, bacteriochlorophyll; Chl, chlorophyll; FMO complex, Fenna−Matthews−Olson complex; fwhm, full width at half-maximum; OPO, optical parametric oscillator; P840, primary electron donor; PMS, *N*-methylphenazonium methosulfate; RCC, reaction center core.

10.1021/bi9910981 CCC: $18.00
Published on Web 09/17/1999

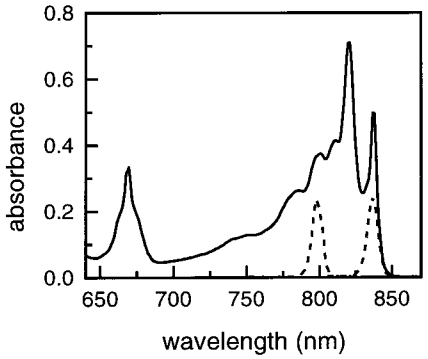

FIGURE 1: Absorption spectrum of the RCC complex at 10 K (solid line) together with the spectra of the narrow band excitation pulses used (dashed lines).

mM potassium phosphate (pH = 6.5) and 2.5 mM Triton X-100. To all samples were added 30 mM sodium ascorbate and 20 μM *N*-methylphenazonium methosulfate (PMS) to keep P840 reduced in the dark. To obtain clear samples at low temperature, glycerol (66% v/v) was added.

Low-temperature time-resolved transient absorption measurements were performed with a home-built amplified dye laser system, operating at 10 Hz, described earlier (*11*, *12*). Excitation pulses were obtained by amplifying the white light continuum in a dye cell. The light passed through a suitable interference filter for narrow-band excitation and an RG780 filter (Schott) for spectrally broad excitation in the near-infrared. LDS 698 and LDS 821 (Exciton) were used for excitation around 670 nm and in the 780−840 nm region, respectively. The time resolution was 300 fs unless otherwise indicated, and the accuracy of the wavelength calibration was ±1 nm. Pump and probe pulses were polarized at the magic angle with respect to each other. All measurements were performed at 10 K, using a helium flow cryostat (Utrecs-LSO, Tartu, Estonia). The concentration of the sample was adjusted to an absorbance of about 0.5 at 837 nm at low temperature; the optical pathway was 0.5 mm.

Millisecond flash-induced absorbance difference kinetics were measured by using a single-beam spectrophotometer (*13*). Excitation flashes were provided by a tunable optical parametric oscillator (OPO) (6 ns pulse duration, 6 nm bandwidth) pumped by a Nd:YAG laser, a xenon flashlamp combined with suitable interference filters (fwhm: 10−12 nm), or the frequency-doubled output of a Q-switched Nd:YAG laser (15 ns pulse duration, 532 nm). The intensity of the excitation flashes was varied by means of neutral density filters and measured with a calibrated photodiode.

RESULTS

Figure 1 shows the low-temperature absorption spectrum of the RCC complex of *P. aestuarii*, together with the profiles of the narrow-band pulses (centered at 799 and 837 nm) used for excitation. Broad band pulses centered at 825 and 672 nm were also used. The absorption spectrum is almost identical to that published earlier (*10*). In the near-infrared region at least six bands can be discerned due to Q_y transitions of BChl *a*, located at 797, 800, 810, 820, 831, and 837 nm, as determined from the second derivative spectrum. Four different bands can be distinguished in the region 660−680 nm corresponding to Q_y transitions of Chl *a* 670 (H. P. Permentier, personal communication).

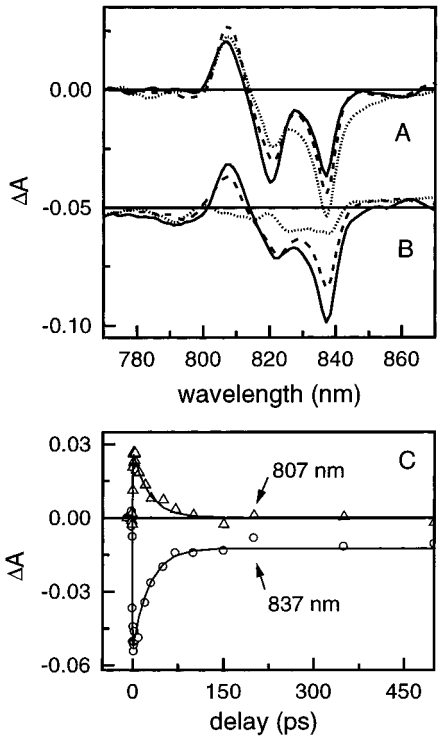

FIGURE 2: (A) Time-resolved difference spectra of the RCC complex at 10 K upon excitation with a spectrally broad pulse (of fwhm 30 nm) centered at 825 nm, at delays of 0.5 ps (solid line), 1.5 ps (dashed line), and 2.5 ps (dotted line) after the onset of the pulse. (B) Spectra at delays of 11 ps (solid line), 21 ps (dashed line), and 500 ps (dotted line). The spectra were plotted with an offset of −0.05 for clarity. The time resolution was 600 fs. (C) Kinetics of absorbance changes at 807 (triangles) and 837 nm (circles). Fits (solid lines) were done with an exponential decay constant of 27 ps and a constant component.

Absorption difference spectra obtained upon broad-band excitation (780−840 nm) at different delays after a pulse are shown in Figure 2. The pulses caused nonselective excitation of essentially all BChl *a* Q_y transitions of the complex. A rapid spectral evolution with time is seen, reflecting downward energy relaxation within the exciton manifold. All spectra show three bands but with different amplitudes. At 0.5 ps after excitation the main bleaching was at 820 nm, but the amplitude of this band decreased rapidly, and already in the difference spectrum at 1.5 ps a band at 837 nm had developed into the largest bleaching band. This band reached its maximum bleaching at 2.5 ps, and subsequently the signal decayed with a time constant of 27 ± 3 ps to a constant value (Figure 2C). The maximum bleaching at 837 nm amounted to 11% of the absorbance at this wavelength; it is obviously due to excited-state formation associated with the transition at 837 nm in the absorption spectrum, together with the generation of stimulated emission from the excited state. The signal amplitude was linear with intensity, indicating that exciton annihilation did not occur. The positive band at 807 nm, which was also observed at room temperature (*11*), may be assigned to excited-state absorption, due to a transition from the singly excited to the doubly excited exciton level. This band is much higher and narrower than that associated with monomeric BChl *a* (*14*, *15*), which is a strong indication for exciton interactions between the BChls (*16*). Similar excited state absorption bands have been observed in difference spectra of the LH2

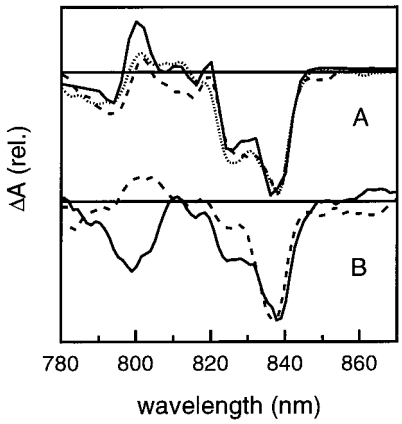

FIGURE 3: Absorption difference spectra of the charge-separated state. (A, solid line) Spectrum obtained with excitation with a 15 ns 532 nm laser pulse. The other spectra were obtained by averaging the signals in the time range of 150−500 ps with various wavelengths of excitation. (A, dashed line) Broad band excitation centered at 825 nm (as in Figure 2) and (A, dotted line) at 672 nm. (B, solid line) Excitation at 799 nm and (B, dashed line) at 837 nm. The amplitudes and positions of the band near 837 nm were normalized.

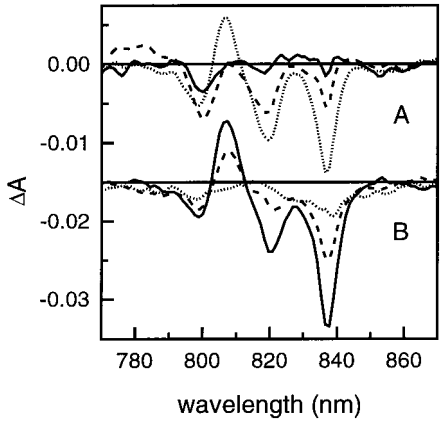

FIGURE 4: Time-resolved spectra of the RCC complex upon excitation at 799 nm: (A) at delays of 0.1 ps (solid line), 0.3 ps (dashed line), and 0.7 ps (dotted line); (B) 2 ps (solid line), 22 ps (dashed line), and 500 ps (dotted line); offset, −0.015.

light-harvesting complex of purple bacteria (*17*, *18*) and of the Fenna−Matthews−Olson (FMO) complex of green sulfur bacteria (*19*, *20*). Model studies with the FMO complex have shown that the oscillator strengths of the various transitions and consequently the shape of the light-induced difference spectra are strongly affected by exciton coupling. Nevertheless, these transitions are dominated by the site energies of the individual pigments and one may therefore associate a particular transition with a single BChl *a* molecule. In view of the overal similarity of the steady-state and excited-state absorption spectra, we assume that the same applies to the RCC complex. We shall therefore in the following discuss spectra in terms of spectral forms of BChl rather than in terms of transitions in the manifold of exciton states.

The spectrum of the constant component is shown in Figure 2B and with better signal-to-noise ratio, obtained by averaging the spectra in the time range of 150−500 ps, in Figure 3. The spectrum lacked the excited-state band at 807 nm and was very similar to that of a 40 ms decay component (Figure 3A, solid line) that has been ascribed to recombination of $P840^+$ with the first iron−sulfur center, F_x^- (*10*). This indicates that the constant component is due to $P840^+$ and that the 27 ps kinetic component reflects the primary charge separation. As noted earlier (*10*), the low-temperature difference spectrum of P840 oxidation is quite complicated, probably due to electrochromic effects of $P840^+$ and changes in exciton interaction. The excited-state difference spectrum that precedes the charge separation did not change appreciably after 2.5 ps, except for amplitude.

Difference spectra obtained upon narrow-band excitation at 799 nm are shown in Figure 4. The difference spectra resemble those earlier obtained at 275 K (*11*), except that the bands are somewhat better resolved. At least three different excited states of BChl *a* can be discerned. The first one was very short-lived and is most clearly seen in the spectrum measured at 100 fs. It consisted of a bleaching at 799 nm, with perhaps some smaller bands at other wavelengths and is apparently mainly due to excitation of BChls *a* 797 and 800. The signal decayed again in about 0.5 ps. A small fraction of the bleaching at 799 nm had a very long lifetime, seen by an additional bleaching on the $P840^+$ difference spectrum (Figure 3B). This effect was also observed at 275 K and was attributed to a small amount of BChl *a* absorbing near 799 nm which is disconnected from the functional BChl *a* (*11*). The other excited states of BChl *a* had also been observed with broad-band excitation (Figure 2). One of these is characterized by a bleaching at 820 nm, which may be ascribed to BChl *a* 820 and which is most prominent in the spectra taken at 0.3 and 0.7 ps. The other one, with a main bleaching band at 837 nm and a strong excited-state absorption at 807 nm, represents the lowest energy level (BChl *a* 837). This state was formed with a time constant of 0.4 ps (not shown). The signals at 837 and 807 nm decayed to a constant value with a time constant of 25 ps, the same as observed upon broad-band excitation. Thus, as at room temperature (*11*), relaxation in the core antenna is more than an order of magnitude faster than charge separation.

Figure 5A,B shows absorbance difference spectra upon excitation at 837 nm. The difference spectrum that formed immediately upon excitation was essentially that of the lowest energy state. The kinetics at 837 nm as well as at 807 nm showed a fast decay component of 1 ± 0.3 ps and a slower one of 22 ± 3 ps (Figure 5C) to a constant value. Figure 3B shows the spectrum of the constant component. Apart from a relatively strong band at 837 nm, it was similar to that obtained with broad-band excitation; we therefore ascribe it to photooxidation of P840. It is of interest to note here that, as at 275 K (*11*), the bleaching band at 837 nm showed a rapid broadening after excitation. The half-width was 4.0 nm initially (0.4 ps); it was 5.3 nm at 0.6 ps, and reached a constant value of 6.2 nm at 6.3 and 13 ps (Figure 6). We conclude that rapid energy transfer occurs between long wavelength BChls with approximately equal energy levels; this process may cause the 1 ps kinetic component mentioned above.

Experiments were also done with a sample that had been cooled in the light. In this case, PMS was omitted and the concentration of ascorbate was lowered to 20 mM. Excitation was again at 837 nm. The excited-state difference spectra (not shown) were essentially the same as those of the previous series, but the difference spectrum at 150−500 ps

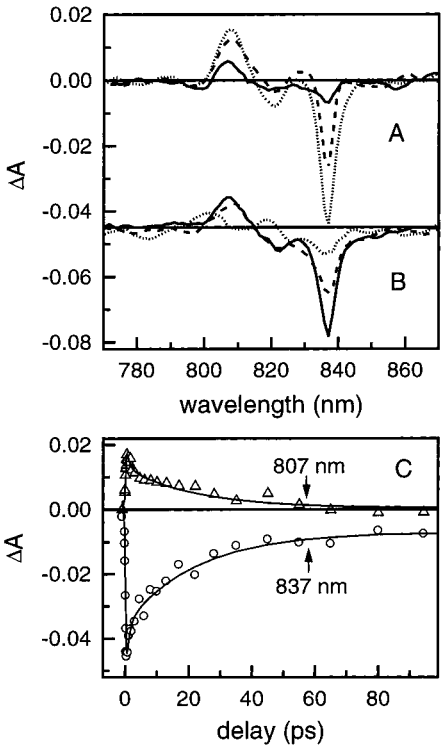

FIGURE 5: (A) Time-resolved spectra of the RCC complex upon excitation at 837 nm at delays of 0.1 ps (solid line), 0.4 ps (dashed line), and 0.6 ps (dotted line). (B) Difference spectra at delays of 6.3 ps (solid line), 22 ps (dashed line), and 500 ps (dotted line); offset, −0.045. (C) Kinetics of absorbance changes at 807 (triangles) and 837 nm (circles). Fits (solid lines) were made with two exponential decay components of 1 and 22 ps and a constant value. The relative amplitudes of the 1 ps, 22 ps, and constant components were 0.61:1:0.27 at 837 nm and 0.55:1:−0.13 at 807 nm, respectively.

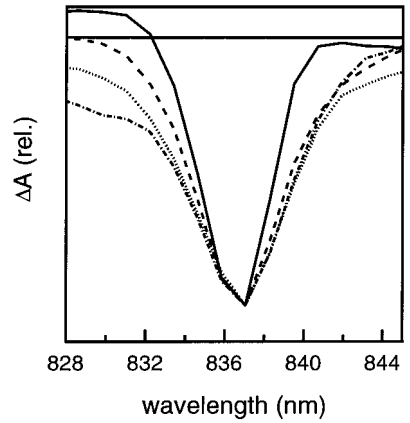

FIGURE 6: Spectra obtained at different delays with excitation at 837 nm, normalized and plotted on an extended scale. Spectra were recorded, in order of increasing bandwidth, at 0.4 ps (fwhm: 4.0 nm), 0.6 ps (fwhm: 5.2 nm), 6.3 ps (fwhm: 6.2 nm), and 13.3 ps (fwhm: 6.3 nm).

showed an about two times smaller bleaching at 837 nm, indicating that at least in part of the reaction centers P840 was in the oxidized state before the pulse. Excited BChl *a* 837 showed a main decay component of 19 ± 3 ps. It thus appears that the excitations are quenched even more strongly by the oxidized than by the "open" reaction center. The same has been observed in heliobacteria (*22*).

Difference spectra and kinetics obtained upon excitation of Chl *a* 670 are shown in Figure 7. The initial spectrum

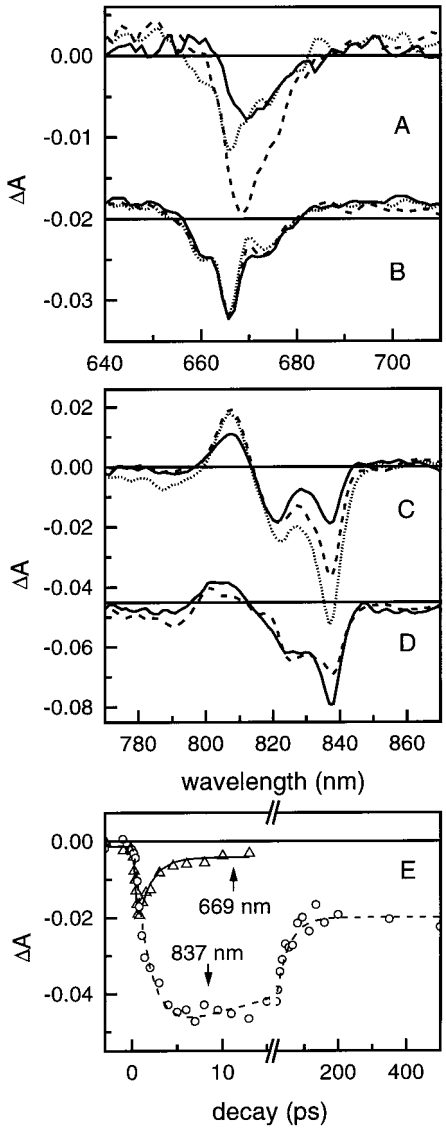

FIGURE 7: Time-resolved spectra upon excitation at 672 nm (fwhm of the excitation pulse: 25 nm): (A) measured at delays of 0.2 ps (solid line), 0.6 ps (dashed line), and 3 ps (dotted line); (B) spectra averaged from 3 to 17 ps (solid line), 22 to 80 ps (dashed line), and 95 to 500 ps (dotted line), offset −0.02; (C) difference spectra recorded in the near-infrared at delays of 0.8 ps (solid line), 2 ps (dashed line), and 7 ps (dotted line); (D) at 35 ps (solid line) and 500 ps (dashed line), offset −0.045; (E) kinetics of absorbance changes upon excitation at 672 nm. Triangles: detected at 669 nm. The fit (solid line) was done with a decay constant of 1.5 ps and a constant value. Circles: Kinetics at 837 nm, fitted (dashed line) with a rise constant of 1.5 ps, a decay of 25 ps, and a constant value. The time resolution was 600 fs.

showed a bleaching centered at 669 nm (Figure 7A). The band developed within the time resolution of the apparatus; its shape was the same as that of the absorption band, indicating that all four spectral forms of Chl *a* 670 were equally excited. Part of the bleaching reversed again with a time constant of 1.5 ps (Figure 7E). The remaining signal was blue-shifted with respect to the initial signal: it appeared to consist of three bleaching bands, at 660 nm (shoulder) and 666 and 675 nm. The amplitude of the signal did not change appreciably after 3 ps and appeared to be stable during at least 1.5 ns.

Difference spectra in the near-infrared upon excitation of Chl *a* 670 are shown in Figure 7C,D. They are not basically

different from those obtained upon broad-band excitation of BChl *a*, with bands at 837, 820, and 807 nm, the last one positive. This indicates that at least part of the excitations on Chl *a* 670 are rapidly transferred to BChl *a* and in fact the bleaching at 837 nm developed with a time constant of 1.5 ps, the same as observed in the decay at 669 nm (Figure 7E). Nevertheless, comparison of the difference spectra at 0.8, 2, and 7 ps indicates that some of the excitations are not transferred directly to BChl *a* 837 but via one or more BChl *a* transitions with intermediate energies. The bleaching at 837 nm decayed with the usual time constant of 27 ± 3 ps to a constant level. The spectrum of the constant component, averaged over the time range 160−500 ps, is plotted in Figure 3A (dotted line). Its shape is very similar to the spectrum obtained with broad-band excitation in the near-infrared and is obviously due to $P840^+$. However, even a cursory inspection of Figure 7C,D shows that the amplitude of the $P840^+$ signal is unusually large as compared to the maximum amplitude of the signal of excited BChl *a* 837. This phenomenon can also be seen in similar difference spectra obtained at 275 K (Figure 4 of ref. *11*). We investigated this effect in a more quantitative way by comparing the amplitude of the constant component with that of the decay component of about 25 ps at 837 nm. It turned out that upon excitation in the near-infrared region (799 nm, 837 nm, or broad-band) the ratio of these two amplitudes varied between 26 and 29%, whereas that upon excitation at 672 nm was 66%.

The above observations would seem to indicate that approximately half of the excitations on Chl *a* 670 are processed in an alternative, direct pathway for charge separation, not involving excited BChl *a* 837. Possible mechanisms will be discussed in the next section. However, we first wanted to exclude a more trivial explanation, namely that significant loss of energy would occur upon excitation of BChl *a* but not of Chl *a* 670. Although our preparation was considerably more active photochemically than most or all other preparations of similar composition described in the literature (*10*), damage during the isolation procedure could not be excluded, and the observation of long-lived excited and strongly fluorescing spectral forms of BChl *a* (Figures 4 and 5; H. P. Permentier, personal communication) could support this notion. Therefore, we compared the relative efficiencies for light absorbed by Chl *a* 670 and by the various spectral forms of BChl *a* in bringing about charge separation, as shown in the experiments of Figure 8. The extent of charge separation was measured as the amount of $P840^+$ decaying with a time constant of 40 ms at 10 K (*10*). Light-induced absorbance changes at 837 nm were measured with various excitation wavelengths as a function of the relative number of absorbed photons. The measurements were done with two different excitation sources. Experiments of Figure 8A were performed with an OPO laser and of Figure 8B with a xenon flashlamp using different wavelengths of excitations in the Q_x and Q_y absorption bands of the BChls *a* (at 600, 800, 820, and 835 nm) and in the Chl *a* 670 band (at 670 nm). Figure 8A,B indicates that the amplitude of the signal at 837 nm divided by the number of absorbed photons is independent of the wavelength of excitation. We conclude that for a given number of absorbed photons the amount of charge separation ($P840^+$) is the same for excitation in the Chl *a* 670 and in the various forms of

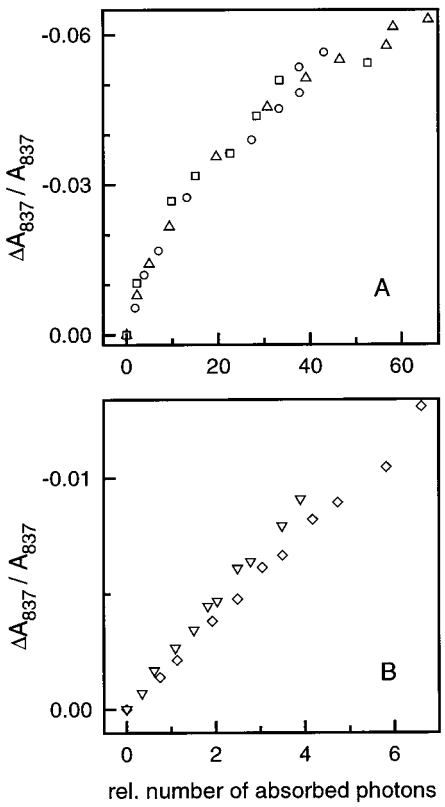

FIGURE 8: Amount of $P840^+$ decaying with a time constant of 40 ms at 10 K, measured upon various excitation wavelengths as a function of the relative number of absorbed photons. (A) Excitation with OPO: 800 nm (circles); 820 nm (up triangles); and 835 nm (squares). (B) Excitation with a xenon flashlamp: 600 nm (down triangles); 670 nm (diamonds).

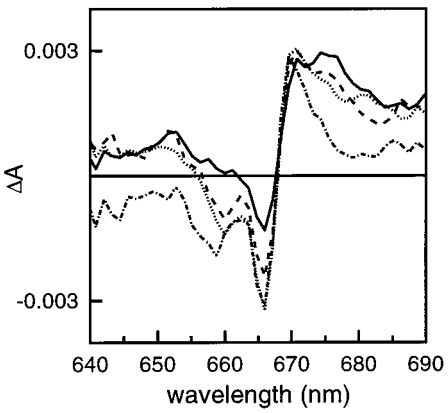

FIGURE 9: Absorbance difference spectra in the Chl *a* 670 region upon excitation of BChl *a* with a spectrally broad pulse, centered at 825 nm (as in Figure 2). Signals were averaged from 1 to 6 ps (solid line), 7 to 27 ps (dashed line), 33 to 153 ps (dotted line) and 203 to 500 ps (dot−dashed line).

BChl *a*. If anything, Chl *a* 670 appears to be somewhat less efficient than BChl *a*. Thus, our observations indicate that excitation of Chl *a* 670 provides a direct way to bring about charge separation without involving excited BChl *a*; there is no significant loss of energy upon excitation of the BChls *a* as the fluorescence experiments might suggest.

The absorbance difference spectra in the Chl *a* 670 region were dependent on the wavelength of excitation. Figure 9 shows spectra obtained upon broad-band excitation of BChl *a*. The spectra, with a negative band below 670 nm and a positive signal at longer wavelengths, resemble those earlier

obtained at longer times after a flash (*7*, *12*) or with continuous illumination (*23*). However, the spectra measured shortly after the pulse showed a much broader absorption increase at wavelengths longer than 670 nm. The development of the difference spectra with time showed various kinetic components. The positive and negative signals at 670 and 666 nm, respectively, formed relatively fast with a time constant of 4 ps, but other features developed more slowly. At intermediate times a negative band near 660 nm evolved, the negative band at 666 nm considerably strengthened, and finally the positive band at 670 nm sharpened, so that in this respect the difference spectrum measured after 200 ps more closely resembled that measured in the millisecond and second range (*21*, *23*). Note that the bands at 660 and 666 nm are also present in the difference spectra of Figure 7B, but with larger amplitude. We tentatively attribute them to reduction of the primary electron acceptor.

DISCUSSION

Our experiments, like those earlier done at 275 K (*11*), clearly indicate that the BChls *a* of the RCC complex form an exciton coupled system. The lowest exciton level is characterized by a bleaching at 837 nm, a weaker negative band at 820 nm, and a positive band, signifying excited-state absorption, at 807 nm. Excited states with higher energy, formed upon short-wavelength excitation, relax at rates which appear to be about the same as those measured at 275 K (*11*): within 2 ps essentially all energy is located at the lowest excited-state level. As discussed earlier, we assume that this state is mainly associated with a discrete BChl *a* species, BChl *a* 837. Excited BChl *a* 837 decays in about 25 ps, and the resulting difference spectrum is clearly due to the charge-separated state, with a bleaching band reflecting the presence of the oxidized primary electron donor, $P840^+$.

The rate at which $P840^+$ formed after a pulse was found to be remarkably independent of temperature (*9*, *11*). This rate can be interpreted in two ways, depending on the trapping model used. In the diffusion-limited model, which was assumed to apply to purple bacteria (*24*, *25*), the rate of charge separation is determined by the rate of energy transfer to the reaction center, and since energy transfer between the core antenna BChls is quite fast, the rate-limiting step in our case would be energy transfer from BChl *a* 837 to P840, i.e., the so-called transfer-to-trap-limited model (*25*) would be valid.

In the trap-limited model excited P840 would be in rapid equilibrium with excited BChl *a* 837 (and at higher temperature also with other BChls), and the rate of conversion of excited P840 to $P840^+$ would be determined by the rate constant for charge separation and the equilibrium constant between excited P840 and core antenna BChls. In this case, the 25 ps decay of the bleaching at 837 nm would contain a significant contribution of excited P840, whereas in the diffusion-limited model this contribution may be very small.

It is of interest to compare our results with those obtained by Nowak et al. (*26*) by means of accumulated photon echo measurements at 1.2 K. Decays observed at 837 nm included components of 1.5, 22, and about 300 ps. Faster components were dominating the kinetics at shorter wavelengths. The 300 ps component can only be of minor quantitative importance, whereas the other two are in good agreement with our measurements. The 22 ps component may be ascribed to charge separation, but since the photon echo technique measures the lifetime of the initially excited state, this would imply that this number represents the true time constant for this process. In other words, the transfer-to-trap-limited model would apply.

On basis of earlier evidence (*3*, *4*, *7*), it has been assumed that Chl *a* 670 is the primary electron acceptor. In this connection it may be remarked that absorption and CD spectra (H. P. Permentier, personal communication) indicate that the protein structure near the Chl *a* 670 binding sites is highly conserved between *P. aestuarii* and *Chlorobium tepidum*, much more so than in the BChl *a* region (*27*), suggesting an essential role for Chl *a* 670 in photochemistry. However, in contrast to observations with the related reaction center of heliobacteria, where low-temperature illumination produces a clear-cut and simple difference spectrum with a bleaching band at 665 nm (*28*), the results obtained by us with the RCC complex of *P. aestuarii* are more complicated. As Figure 9 shows, excitation in the BChl *a* region first produced a spectrum with a negative band at 666 nm and a broad positive band at 670−700 nm. This spectrum was formed rapidly (4 ps), and we have no ready explanation for this phenomenon. Then an additional bleaching occurs with bands at 660 and 666 nm which could well be explained by a reduction of Chl *a* 670 resulting from the charge separation, but the amplitude is relatively small, as was also observed at 275 K (*11*). It should be kept in mind, however, that the negative band produced upon photoreduction of bacteriopheophytin in the reaction center of purple bacteria is quite weak too (*29*). Finally, in the time range of several hundreds of picoseconds, the positive band at 670 nm sharpens so that the resulting spectrum more closely resembles the spectrum measured at later times (*21*). At the same time the negative band at 660 nm appears to shift to shorter wavelengths. This spectral change might signify a transfer of electrons to a secondary electron acceptor.

Illumination at 672 nm produced a larger $P840^+$ signal relative to that of excited BChl *a* 837 (Figure 7). This indicates the presence of an additional pathway for charge separation. To be efficient, the initial step of this process should occur with approximately the same time constant as that for energy transfer to BChl *a*, together giving an 1.5 ps exponential decay of excited Chl *a* 670. Two possible mechanisms for this additional pathway may be envisaged: (i) Direct energy transfer occurs from excited Chl *a* 670 to P840, followed by charge separation, $P840^* A_0 \rightarrow P840^+ A_0^-$. Such a scheme, of course, would require that the trap-limited model for energy transfer does not apply, since otherwise the excitations would diffuse back from excited P840 to BChl *a* 837. (ii) Charge separation occurs by a process involving Chl *a* 670 as a primary photochemical reactant, e.g. by a reaction (assuming Chl *a* 670 to be identical to A_0) $P840 A_0^* \rightarrow P840^+ A_0^-$ analogous to what has been proposed by van Brederode et al. (*30*, *31*) for purple bacteria. Both schemes appear to be compatible with our experimental results and are supported by the difference spectra obtained in the 670 nm region, where a negative signal is observed with bands at 660, 666, and 670 nm which shows hardly any decay in the time range 10−1500 ps. This suggests that the bleaching is due to a chemical intermediate

rather than an excited state of Chl *a* 670. Both schemes would imply a low yield of BChl *a* fluorescence upon excitation of Chl *a* 670, but this effect would be hard to observe because of the presence of strongly fluorescing BChl *a* pools, mentioned earlier. It may be noted here that precisely this effect was observed in *Heliobacterium chlorum* (*32*), suggesting that the "additional pathway" for charge separation does also exist in heliobacteria. This point is presently under investigation in our laboratory.

ACKNOWLEDGMENT

The authors thank H. P. Permentier for stimulating discussions and for providing us with the low-temperature absorption spectrum of the RCC complex.

REFERENCES

1. Feiler, U., and Hauska, G. (1995) in *Anoxygenic Photosynthetic Bacteria* (Blankenship, R. E., Madigan, M. T., and Bauer, C. E., Eds.) pp 665−685, Kluwer Academic Publishers, Dordrecht, The Netherlands.
2. Permentier, H. P., Schmidt, K. A., Francke, C., Neerken, S., Hager-Braun, C., and Amesz, J. (1998) in *Photosynthesis: Mechanisms and Effects* (Garab, G., Ed.) pp 527−530, Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. Nuijs, A. M., Vasmel, H., Joppe, H. L. P., Duysens, L. N. M., and Amesz, J. (1985) *Biochim. Biophys. Acta 807*, 24−34.
4. Shuvalov, V. A., Amesz, J., and Duysens, L. N. M. (1986) *Biochim. Biophys. Acta 851*, 1−5.
5. van de Meent, E. J., Kobayashi, M., Erkelens, C., van Veelen, P. A., Otte, S. C. M., Inoue, K., Watanabe, T., and Amesz, J. (1992) *Biochim. Biophys. Acta 1102*, 371−378.
6. Krauss, N., Schubert, W.-D., Klukas, O., Fromme, P., Witt, H. T., and Saenger, W. (1996) *Nat. Struct. Biol. 3*, 965−973.
7. van Kan, P. J. M. (1991) Doctoral Thesis, Leiden University.
8. Kramer, H., Aartsma, T. J., and Amesz, J. (1996) *Photochem. Photobiol. 64*, 26−31.
9. Oh-oka, H., Kamei, S., Matsubara, H., Lin, S., van Noort, P. I., and Blankenship, R. E. (1998) *J. Phys. Chem. B 102*, 8190−8195.
10. Francke, C., Permentier, H. P., Franken, E. M., Neerken, S., and Amesz, J. (1997) *Biochemistry 36*, 14167−14172.
11. Neerken, S., Permentier, H. P., Francke, C., Aartsma, T. J., and Amesz, J. (1998) *Biochemistry 37*, 10792−10797.
12. Kennis, J. T. M. (1997) Doctoral Thesis, Leiden University.
13. Franken, E. M., and Amesz, J. (1997) *Biochim. Biophys. Acta 1319*, 214−222.
14. Becker, M., Nagarajan, V., and Parson, W. W. (1991) *J. Am. Chem. Soc. 113*, 6840−6848.
15. Musewald, C., Hartwich, G., Pollingerdammer, F., Lossau, H., Scheer, H., and Michel-Beyerle, M. E. (1998) *J. Phys. Chem. B 102*, 8336−8342.
16. van Burgel, M., Wiersma, A. D., and Duppen, K. (1995) *J. Chem. Phys. 102*, 20−33.
17. Kennis, J. T. M., Streltsov, A. M., Permentier, H. P., Aartsma, T. J., and Amesz, J. (1997) *J. Phys. Chem. 101*, 8369−8374.
18. Stiel, H., Leupold, D., Teuchner, K., Nowak, F., Scheer, H., and Cogdell, R. J. (1997) *Chem. Phys. Lett. 276*, 62−69.
19. Vulto, S. I. E., Neerken, S., Louwe, R. J. W., de Baat, M. A., Amesz, J., and Aartsma, T. J. (1998) *J. Phys. Chem. B 102*, 10630−10635.
20. Savikhin, S., Buck, D. R., and Struve, W. S. (1998) *J. Phys. Chem. B 102*, 5556−5565.
21. Schmidt, K. A., Permentier, H. P., de Wijn, R., Neerken, S., Franken, E. M., Francke, C., Hager-Braun, C., Aartsma, T. J., and Amesz, J. (1998) in *Photosynthesis: Mechanisms and Effects* (Garab, G., Ed.) pp 543−546, Kluwer Academic Publishers, Dordrecht, The Netherlands.
22. Deinum, G., Kramer, H., Aartsma, T. J., Kleinherenbrink, F. A. M., and Amesz, J. (1991) *Biochim. Biophys. Acta 1058*, 339−344.
23. Swarthoff, T., van der Veek-Horsley, K. M., and Amesz, J. (1981) *Biochim. Biophys. Acta 635*, 1−12.
24. Otte, S. C. M., Kleinherenbrink, F. A. M., and Amesz, J. (1993) *Biochim. Biophys. Acta 1143*, 84−90.
25. Timpmann, K., Freiberg, A., and Sundström, V. (1995) *Chem. Phys. 194*, 275−283.
26. Nowak, F. R., Louwe, R. J. W., Permentier, H. P., Schmidt, K. A., and Aartsma, T. J. (1998) in *Photosynthesis: Mechanisms and Effects* (Garab, G., Ed.) pp 185−188, Kluwer Academic Publishers, Dordrecht, The Netherlands.
27. Francke, C., and Amesz, J. (1997) *Photosynth. Res. 52*, 137−146.
28. van Kan, P. J. M., Aartsma, T. J., and Amesz, J. (1989) *Photosynth. Res. 22*, 61−68.
29. Shuvalov, V. A., and Parson, W. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 957−961.
30. van Brederode, M. E., Jones, M. R., van Mourik, F., van Stokkum, I. H. M., and van Grondelle, R. (1997) *Biochemistry 36*, 6855−6861.
31. van Brederode, M. E., van Mourik, F., van Stokkum, I. H. M., Jones, M. R., and van Grondelle, R. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 2054−2059.
32. van de Meent, E. J., Kleinherenbrink, F. A. M., and Amesz, J. (1990) *Biochim. Biophys. Acta 1015*, 223−230.

BI9910981