Pathways of Energy Transformation in Antenna Reaction Center Complexes of *Heliobacillus mobilis*†

Sieglinde Neerken,* Thijs J. Aartsma, and Jan Amesz

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

Received October 20, 1999; Revised Manuscript Received December 28, 1999

ABSTRACT: The conversion of excitation energy in the antenna reaction center complex of *Heliobacillus mobilis* was investigated at 10 K as well as at 275 K by means of time-resolved absorbance difference spectroscopy of isolated membranes in the (sub)picosecond time range. Selective excitation of the primary electron acceptor, chlorophyll (Chl) *a* 670, and of the different spectral pools of bacteriochlorophyll (BChl) *g* (BChl *g* 778, BChl *g* 793, and BChl *g* 808) was applied. At 10 K, excitation at 770 or 793 nm resulted on the one hand in rapid energy transfer to BChl *g* 808 and on the other hand in fast charge separation from excited BChl *g* 793 (∼1 ps). Once the excitations were on BChl *g* 808, the bleaching band shifted gradually to the red, from 806 to 813 nm, and charge separation from excited BChl *g* 808 occurred by a very slow process (∼500 ps). The main purpose of our experiments was to answer the question whether an "alternative" pathway for charge separation exists upon excitation of Chl *a* 670. Our measurements showed that the amount of oxidized primary donor ($P798^+$) relative to that of excited BChl *g* produced by excitation of Chl *a* 670 was considerably larger than upon direct excitation of BChl *g*. This indicates the existence of an alternative pathway for charge separation that does not involve excited antenna BChl *g*. This effect occurred at 10 K as well as at 275 K. The mechanism for this process is discussed in relation to different trapping models; it is concluded that charge separation occurs directly from excited Chl *a* 670.

The traditional scheme for the primary processes in photosynthesis involves (i) the absorption of light by antenna pigments, (ii) transfer of excitation energy to the primary electron donor, and (iii) the transfer of an electron from the primary donor to an acceptor molecule.

However, there are several aspects to this scheme that are the subject of discussion. First of all, it is not always clear whether step ii or step iii is rate-limiting in the generation of the charge-separated state, and both possibilities have, e.g., been considered for purple bacteria (*1*, *2*). Second, the mechanism for so-called uphill transfer of excitation energy from the antenna to the reaction center, which appears to occur even at liquid helium temperature in heliobacteria and various species of purple bacteria (*3*, *4*), is not understood. Finally, it has been proposed that alternative pathways may exist for charge separation that do not involve the excited state of the primary electron donor (*4*−*7*).

Recently we performed time-resolved studies of the excited states and charge separation in reaction center core complexes of the green sulfur bacterium *Prosthecochloris (Ptc.) aestuarii* (*7*, *8*). These complexes contain about 16 bacteriochlorophylls (BChl)[1] *a*, two of which form the special pair P840, and four chlorophyll (Chl) *a* molecules absorbing near 670 nm (Chl *a* 670) (*9*, *10*). Comparison of the population of excited BChl *a* and the extent of subsequent charge separation brought about by excitation either of BChl *a* or of Chl *a* 670 showed that upon excitation of the latter pigment an alternative pathway of charge separation existed. The phenomenon appeared to occur at 10 K as well as at 275 K.

The question now arises whether a similar pathway might exist in heliobacteria. Like the green sulfur bacteria, the heliobacteria have a type I reaction center (*11*, *12*). In contrast to other photosynthetic organisms they have a single pigment protein complex, which was called the ARC (antenna reaction center) complex (*13*) and which can be isolated after detergent solubilization (*13*, *14*). The visible absorption spectrum of the isolated ARC complex is identical to that of whole cells and cytoplasmic membranes (*13*). The complex contains about 35 molecules of BChl *g* (*15*), a pigment related to BChl *a*, but with an ethylidene group at carbon C8 (ring II) (*16*). It absorbs in the Q_y region with three bands peaking near 778, 793, and 808 nm, which are only resolved at low temperature. The corresponding spectral forms have been called BChl *g* 778, BChl *g* 793, and BChl *g* 808 (*17*). Only for BChl *g* 793 is there evidence for strong excitonic interaction (*17*). The primary electron donor, P798, is a dimer of BChl *g*, presumably of its 13^2 epimer (*18*). The ARC complex also contains two molecules of 8^1-hydroxy Chl *a* (*15*). The latter pigment has a Q_y absorption band at 668 nm and appears to act as primary electron acceptor, A_0 (*19*). Although chemically not identical, we shall call it Chl *a* 670, as in green sulfur bacteria (*8*).

Nuijs et al. (*19*) were the first to apply pump−probe absorption spectroscopy to the study of energy transforma-

† This work was supported by the Section for Earth and Life Sciences (ALW) of The Netherlands Science Foundation (NWO).

* Author to whom correspondence should be addressed: e-mail sigi@biophys.leidenuniv.nl; fax: +31 71 5275819.

[1] Abbreviations: A_0, primary electron acceptor; ARC, antenna reaction center; BChl, bacteriochlorophyll; Chl, chlorophyll; P798, primary electron donor.

10.1021/bi992433o CCC: $19.00
Published on Web 03/03/2000

tions and electron transfer in membranes of heliobacteria, and this has been followed by many other studies during the past decade. The older experiments could not resolve the energy transfer steps in the pigment system, but they demonstrated that charge separation occurs in 20−30 ps at room temperature and showed that the establishment of thermal equilibrium takes at most a few picoseconds (*20*). This was essentially confirmed by more recent measurements with higher time resolution (*21*, *22*).

Results obtained at low temperature were more complicated. As first observed by van Kan et al. (*23*), at low temperature the excitations tend to accumulate on the longest wavelength BChl, BChl *g* 808. Although P798 absorbs at a considerably shorter wavelength, the yield of charge separation, even at 6 K, is still significant (*24*). This reaction would be fairly slow at cryogenic temperature, with a time constant of 30 ps at 140 K and of 70 ps at 20 K (*25*). Recent experiments at 20 K by Liebl et al. (*26*) and by Chiou et al. (*4*) led to partially conflicting conclusions. The latter investigators concluded that there is a rapid equilibration of the excitation energy, followed by charge separation starting from excited BChl *g* 808. However, Liebl et al. observed a much more rapid charge separation from excited BChl *g* 793, which reaction obviously occurred before a thermal equilibrium was established.

In this paper a comparative study is given of transient absorption changes in membranes of *Heliobacillus (Hba.) mobilis*, measured at 275 as well as at 10 K with excitation of either Chl *a* 670 or of BChl *g*. The results indicate that in heliobacteria, like in green sulfur bacteria, a direct pathway for charge separation from excited Chl *a* 670 exists, not involving excited antenna BChl. They confirm the existence of a rapid charge separation from excited BChl *g* 793. When initiated by excited BChl *g* 808, charge separation is very slow, with a time constant of about 500 ps.

MATERIALS AND METHODS

Hba. mobilis was grown as described by van de Meent et al. (*13*) and membrane fragments were prepared essentially as described by Francke et al. (*27*). Sodium ascorbate (10 mM) and 20 μM *N*-methylphenazonium methosulfate (PMS) were added to keep P798 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, as described earlier (*8*, *28*). Excitation pulses were obtained by amplifying the white light continuum in a dye cell. LDS 698, LDS 751, and LDS 821 (Exciton) were used for excitation around 670, 770, and 793 and 811 nm, respectively. To reduce the bandwidth of the excitation pulse, the light was passed through a suitable interference filter. The spectral widths of the excitation pulses were 22 nm for excitation at 668 and 11 nm for excitation of the different spectral forms of BChl *g*, respectively. The time resolution was 300 fs, 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. Measurements at 10 K were performed with a helium flow cryostat (Utrecs-LSO, Tartu, Estonia). The concentration of the sample was adjusted to an absorbance of about 0.8 at 793 nm at low temperature; the optical pathway was 0.5 mm. It was checked that the signals were linear with excitation intensity. Experiments at 275 K were performed in the absence of glycerol with a moving cuvette as described elsewhere (*8*). The absorbance of the sample was 0.9 at 788 nm.

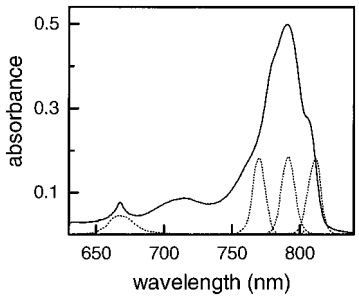

FIGURE 1: Absorption spectrum of isolated membrane fragments from *Hba. mobilis* at 6 K (adapted from ref *27*) together with the profiles of the excitation pulses at 668, 770, 793, and 811 nm (dotted lines).

RESULTS

Absorption Spectrum. Time-resolved absorbance difference spectra were measured with different selective excitations in the Q_y absorption bands of BChl *g* and Chl *a* 670. Figure 1 shows the low-temperature absorption spectrum of isolated membrane fragments from *Hba. mobilis* together with the spectral profiles of the excitation pulses at 668, 770, 793, and 811 nm (dotted lines). The absorption bands of the three different spectral forms of BChl *g* can clearly be identified in the spectrum: a maximum at 794 nm and two shoulders at 779 and 809 nm, as determined from the second derivative (*27*). For convenience we will use the same nomenclature as for *Heliobacterium (Hbt.) chlorum* (*17*) for the different spectral forms of BChl *g*: BChl *g* 778, BChl *g* 793, and BChl *g* 808. The absorption peak at 668 nm is due to Chl *a* 670, the electron acceptor pigment, A_0 (*19*); the Q_x band of BChl *g* (not shown) is located around 576 nm (*27*).

Excitation of BChl g. Transient absorption difference spectra at 10 K were measured with selective excitation of BChl *g* 793. Some of the resulting spectra at different delay times after excitation are shown in Figure 2. In the first spectrum, at 0.1 ps delay, a bleaching band is detected with a maximum located at 794 nm, due to excited BChl *g* 793, together with a small shoulder at 807 nm. The latter, which is obviously due to excited BChl *g* 808, developed into the largest bleaching band in the spectrum, with a time constant of about 1.1 ps (Figure 3). This indicates that excitation energy from BChl *g* 793 is rapidly transferred downhill to BChl *g* 808; the time constant of 1.1 ps is in good agreement with that earlier reported by Liebl et al. (*26*), but smaller than the 2.6 ps constant observed by Chiou et al. (*4*). The maximum bleaching was reached after 4 ps. The band gradually shifted with time to 813 nm and showed a complicated decay pattern, with exponential time constants of 7, 60, and 450 ps (Figure 3). These constants were obtained by a fit of the area integrated between 803 and 818 nm plotted as a function of time. In this way the effects of noise and of the band shift were minimized. The time constant of 7 ps has been ascribed to relaxation within the BChl *g* 808 pool (*26*). The slower component of 60 ps may

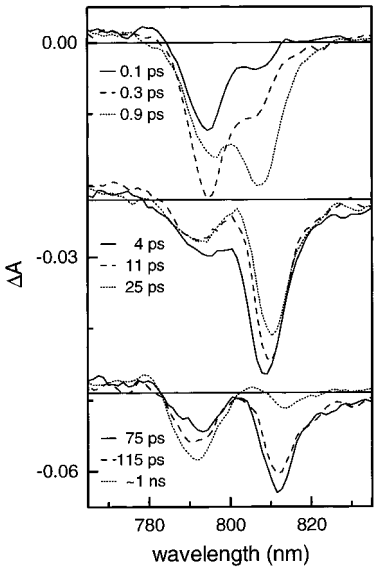

FIGURE 2: Time-resolved difference spectra of membrane fragments from *Hba. mobilis* at 10 K upon excitation at 793 nm at various delays after the onset of the pulse. Spectra measured at 4 ps and longer delays were shifted vertically. The spectrum at ~1 ns gives the signal averaged over the time range of 650−1200 ps.

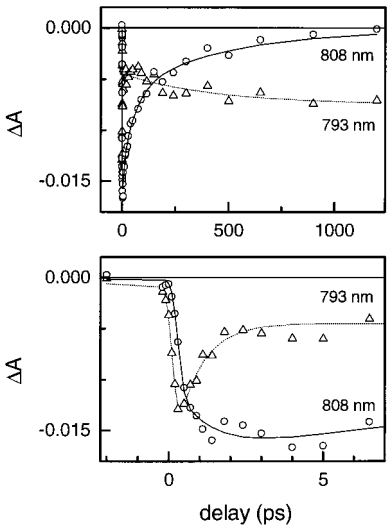

FIGURE 3: Kinetics of absorbance changes upon excitation at 793 nm, detected near 793 nm (△, measured as the area 785−797 nm, see text) and near 808 nm (○, measured at 803−818 nm). Fits were done with the following exponential constants: at 793 nm, decay of 0.80 ps, rise of 425 ps, and a constant component with relative amplitudes 1:−0.24:0.54; at 809 nm, rise of 1.1 ps and decays of 7, 60, and 440 ps with relative amplitudes 0.57:1:0.84.

correspond to the 50−80 ps decay observed earlier (*4*, *23*, *25*, *26*). The 450 ps component has not been observed before.

The 793 nm band decayed with a time constant of 0.8 ps. However, only part of the bleaching decayed in this way. This indicates that not all of the excitations on BChl *g* 793 are transferred to BChl *g* 808. As was argued earlier by Liebl et al. (*26*), part of the excitations appear to result in rapid formation of $P798^+$, which also causes a bleaching band at 793 nm at low temperature (*29*). To be efficient, this process of charge separation must occur at about the same rate as energy transfer to BChl *g* 808. No evidence was seen for a 60−70 ps component of charge separation earlier reported by Lin et al. (*25*): the amplitude of the bleaching band at 793 nm was essentially constant between 10 and 75 ps

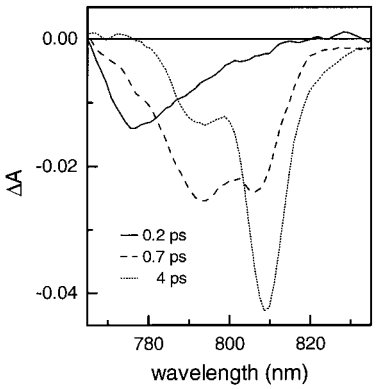

FIGURE 4: Time-resolved difference spectra at 10 K upon excitation at 770 nm at delays indicated.

(Figure 2). However, at still later times it showed a significant increase, indicating a very slow process of charge separation, which may be related to the 450 ps decay component of excited BChl *g* 808. As Figures 2 and 3 show, the amplitude at 793 nm increased between 75 ps and 1.2 ns by approximately a factor of 1.5. We conclude that charge separation occurs in at least two ways at low temperature: from excited BChl *g* 793 by a fast (~1 ps) and from excited BChl *g* 808 by a very slow (~450 ps) process; the first one occurs without prior equilibration of the excitations within the ARC complex and competes with energy transfer to BChl *g* 808.

Difference spectra obtained with excitation at 770 nm are shown in Figure 4. In this case the BChl *g* 778 pool was excited, resulting in a bleaching band at 776 nm. However, at 0.2 ps the band was already clearly asymmetric and appeared to contain contributions by excited BChl *g* 793 and BChl *g* 808, indicating that the excitation energy was rapidly transferred to the BChl *g* pools with lower energies. The band at about 808 nm developed with a time constant of 0.50 ps, more rapidly than with 793 nm excitation, apparently by energy transfer from BChl *g* 778 to BChl *g* 808 (*26*). At 0.7 ps the spectrum was dominated by negative peaks at 793 and 808 nm. After 4 ps, the difference spectra became virtually identical to those obtained with 793 nm excitation. The BChl *g* 808 bleaching band decayed with time constants of 7, 50, and 500 ps (relative amplitudes 0.17:1:0.47), and the latter was again accompanied by a very slow increase of the bleaching at 793 nm (not shown). The maximum bleaching shifted with time from 806 to 813.5 nm, as was earlier observed upon excitation at 793 nm. This shift is shown in Figure 5. An analysis of the temporal profile of the peak position of the BChl *g* 808 band yielded two time constants of 2.3 and 123 ps with about equal amplitudes. A satisfactory fit could not be obtained with the time constants used for the decay kinetics. This indicates that there is no simple relation between the position of the band and its rate of decay.

Figure 6 shows difference spectra with excitation at 811 nm, at the red side of the absorption spectrum. Initially, the spectra only showed excited states of BChl *g* 808. A single bleaching was measured at 809 nm, with a half-width of 7 nm. The band broadened rapidly on the red side, attained a width of 9 nm, and reached its maximum amplitude within less than 0.5 ps. In the subsequent spectra the amplitude of the band decreased and the peak eventually shifted to 813

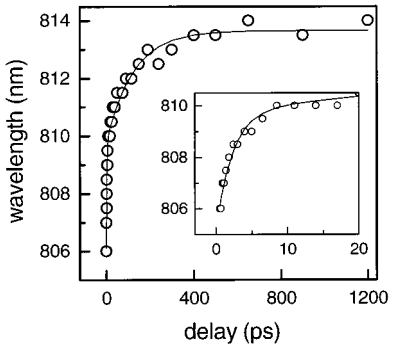

FIGURE 5: Position of the maximum of the long-wavelength bleaching with time upon excitation at 770 nm. The line shows a fit with two exponential components, 2.3 and 123 ps, with relative amplitudes 1:0.85. Inset: Data on an extended time scale.

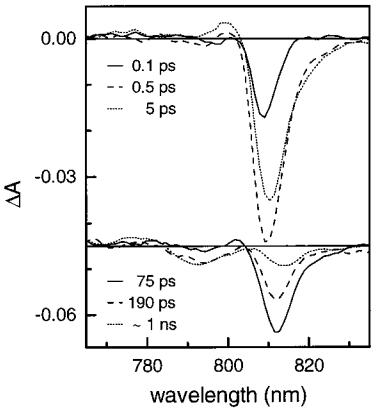

FIGURE 6: Time-resolved difference spectra at 10 K upon excitation at 811 nm at delays indicated.

nm, as in the spectra described above. The best fit of the amplitude of the band was obtained with decay constants of 4.2, 75, and 600 ps and relative amplitudes of 0.54:1:0.37 (not shown), but a reasonable fit was also obtained with the same constants as in Figure 3. As in the experiments described above, only a very slow photooxidation of P798 was observed.

Excitation of Chl a 670. Time-resolved absorbance difference spectra in the near-infrared were also measured with excitation at 668 nm (Figure 7A). The maximum of the excitation pulse coincided with the Q_y absorption maximum of Chl *a* 670 (Figure 1). However, BChl *g* also is excited directly at this wavelength; as suggested by the absorption spectrum, a considerable part of the absorption near 670 nm must arise from vibronic bands of BChl *g*. Therefore, a quite complicated kinetic behavior might be expected. As Figure 7 shows, a rapid spectral evolution with time was seen. At 200 fs delay (Figure 7A), two peaks appeared in the spectrum, the largest one being located at 794 nm and the smaller one at 807 nm. At 600 fs this situation was reversed (not shown). The maxima of the bleaching bands were reached at delays of 1.1 ps for the band around 794 nm and at 2 ps for the band around 807 nm. For the latter one, the kinetics showed a rise component of 0.6 ps. It is not clear if this reflects energy transfer from Chl *a* 670 or from the other BChls *g* to BChl *g* 808. In contrast to Chiou et al. (*4*), we observed a rapid rise at 793 nm after the pulse, and in fact our spectrum at 200 fs was very similar to their spectrum at 800 fs. This rise was close to the time resolution of our apparatus: we can only conclude that energy transfer from

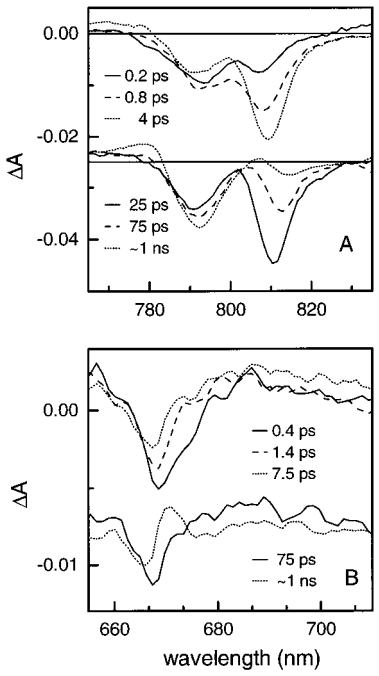

FIGURE 7: Time-resolved difference spectra at 10 K upon excitation at 668 nm at the delays indicated, measured in the BChl *g* region (A) or in the region of Chl *a* 670 (B). The spectra at 75 ps and ~1 ns in panel B were plotted with an offset of −0.008.

Chl *a* 670 to BChl *g* 793, if it occurs, must be faster than 0.5 ps. A bleaching band at 779 nm is not observed upon excitation at 668 nm. This may indicate that almost no excitation energy is transferred to BChl *g* 778. With one important difference (see below), the spectra measured after a delay of a few picoseconds resembled those obtained upon excitation at 793 nm. Again, a fast component (~1 ps) in the decay of the 793 nm band was observed, which is ascribed to a combination of charge separation from excited BChl *g* 793 and energy transfer to BChl *g* 808. The long-wavelength band shifted with time to 813 nm; its amplitude showed decay constants of 60 and 450 ps, as in the experiments of the previous section, but in this case a 7 ps decay component was not observed. In Figure 7A it can be seen that the amplitude of the band at 793 nm increased at a very long time scale, reflecting charge separation from excited BChl *g* 808.

From a quantitative comparison of the spectra measured upon excitation of Chl *a* 670 with those upon excitation of BChl *g* 793 (Figure 2), it is obvious that the amount of $P798^+$ produced upon 668 nm excitation is relatively large compared to that of excited antenna BChl *g*. This effect is seen even more clearly in Figure 8, which compares at two different delays the difference spectra for both kinds of excitation. The amount of $P798^+$ produced relative to the number of excited states generated in BChl *g* was almost 50% larger upon excitation of Chl *a* 670, at 5 ps after the pulse as well as at 1 ns, when charge separation was essentially completed. In *Hbt. chlorum* a low yield of BChl *g* fluorescence upon excitation of Chl *a* 670 was observed (*13*), suggesting that under those conditions a direct pathway for charge separation exists in heliobacteria. The existence of such a pathway now appears to be confirmed by our time-resolved experiments. Similar results were obtained by us with reaction center core complexes of green sulfur bacteria (*7*) and have likewise been ascribed to a pathway of charge separation operating directly

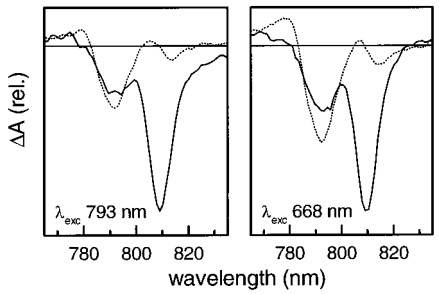

FIGURE 8: Time-resolved difference spectra at 10 K upon excitation at 793 or 668 nm. Solid lines, spectra at 5 ps delay; dotted lines, spectra at 650−1200 ps. The spectra were normalized at the maximum at 808 nm at 5 ps.

from excited Chl *a* 670. Like for green sulfur bacteria, two possible schemes can explain this direct step of charge separation from the excited acceptor pigment, Chl *a* 670 (A_0). According to the first one the excitations on Chl *a* 670 are directly transferred to P798 and subsequently charge separation occurs from excited P798. In the second scheme, charge separation occurs from the excited acceptor pigment in a reaction not involving excited P798, e.g., by the reaction P798 Chl *a* 670* → $P798^+$ Chl *a* 670^-.

The formation of the charge-separated state can also be observed in the region of the primary electron acceptor. The first spectra upon excitation of Chl *a* 670 showed a bleaching band at 668 nm, which we ascribe to excited Chl *a* 670 (Figure 7B). The signal is superimposed on a broad positive signal that may be ascribed to excited BChl *g* (*23*). Part of the bleaching decayed rapidly, while the band sharpened, decreased in amplitude and shifted slightly to the blue. This may be ascribed to the reduction of A_0 and perhaps to energy transfer to BChl *g*. The spectra measured at 1.4 and 7.5 ps are similar to those reported earlier upon excitation at 668 nm (*4*), while those measured at later times resemble those obtained by van Kan (*30*). The decay pattern presumably reflects electron transfer from A_0 to the next electron acceptor, with a time constant of 300 ps (*23*). In agreement with this, at later times (about 1 ns, Figure 7B) the difference spectrum changed into one with a bleaching at 666 and a positive band at 670 nm, which can be ascribed to an electrochromic shift of Chl *a* 670 caused by the charge separated state.

Experiments at 275 K. The existence of the additional pathway for charge separation described above was even more evident at higher temperature. Figure 9 shows some difference spectra obtained at 275 K upon excitation at 770 or 668 nm. With 770 nm excitation, the initial spectrum showed a bleaching band at 775 nm, but the maximum rapidly shifted to longer wavelengths, and at 1.4 ps a thermal equilibrium was reached with a maximum bleaching at 805 nm. Upon excitation at 668 nm, the difference spectrum initially showed a broad bleaching around 800 nm. This was again followed by a red shift, and after 1.4 ps the spectrum was similar to that obtained upon excitation at 770 nm. An important difference, however, was in the extent of charge separation. Since the bleaching band shows no structure at 275 K, a comparison of the amplitudes is more straightforward and much easier than for the low-temperature experiments. Figure 9 panels A and B compare the amount of $P798^+$ relative to the amplitude of the maximum signal of excited BChl *g* for excitation at 770 and 668 nm, respectively. The relative bleaching at about 1 ns, which we ascribe to $P798^+$, was a factor of 1.5 larger with excitation of Chl *a* 670 than of BChl *g*. This factor is the same as was found in the experiments performed at 10 K.

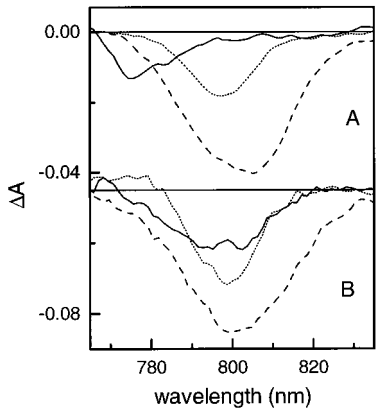

FIGURE 9: Time-resolved difference spectra at 275 K upon excitation at (A) 770 and (B) 668 nm. (A) Signals at delays of 0.1 ps (solid line), 1.4 ps (dashed line), and 650−1200 ps (dotted line). (B) Signals at delays of 0.2 ps (solid line), 1.4 ps (dashed line), and 650−1200 ps (dotted line). The spectra were normalized at their maxima at 1.4 ps.

DISCUSSION

Our results confirm the observation of Liebl et al. (*26*) regarding the existence of a fast charge separation from excited BChl *g* 793 at low temperature. Thus, the model proposed by Chiou et al. (*4*), whereby a thermal equilibrium is established before charge separation takes place, is not confirmed by our observations. Charge separation, or energy transfer to P798, competes with energy transfer to BChl *g* 808 with a time constant for the combined reaction of about 1 ps, implying that the charge separation is about as fast as in purple bacteria (*31*).

The rapid phase of charge separation is not observed at room temperature. Most investigators report a single time constant of 20−30 ps at room temperature (20, 21), but Liebl et al. (*22*) observed in addition a 5 ps component as well. No matter whether the reaction is monophasic or biphasic, it is hard to imagine that the 1 ps reaction would only occur at cryogenic temperature. This then strongly suggests that the apparent absence of this reaction must be explained by the assumption that at room temperature the trap-limited model for charge separation (or a situation that approaches the trap-limited model) applies, whereby excited P798 is in thermal equilibrium with the antenna BChls *g*, so that the rate of charge separation depends on both the rate constant of charge separation and the effective size of the equilibration pool. This has important implications for the mechanism of the "alternative" pathway of charge separation as will be discussed below. It may be noted that the trap-limited model was used by Lin to calculate a "true" rate constant of charge separation of $(1.2\ ps)^{-1}$ from the observed time constant of 27−30 ps (*21*).

A second point of interest is the decay of the excited state of BChl *g* 808. Three decay constants were obtained, of about 7, 60, and 500 ps. In our hands, the first one was quite variable; its relative amplitude was different for different excitation conditions, and also the value of the time constant varied. It has been ascribed to energy relaxation within the

BChl *g* 808 pool. Such a decay component may signify a change in oscillator strength rather than in the number of excitations. This change may be due to relaxation in the exciton-coupled system of excited states. The two other components are much more reproducible. The 60 ps decay component has been repeatedly observed (*4*, *23*, *25*, *26*, *30*). According to Lin et al. (*25*), this component would be coupled to charge separation. This was not confirmed by our measurements, although admittedly the noise level of our experiments would not allow the observation of a fairly small signal. It may be noted here that most of the so-called decay-associated spectra of Chiou et al. (*4*) and Liebl et al. (*26*) do not support this notion either, and Liebl et al. in fact ascribed the 60 ps decay to quenching by $P798^+$. We have no evidence that it is due to excitation annihilation coupled to energy transfer between different ARC complexes. Not only was the extent of charge separation linear with the excitation intensity, but also the decay of the BChl *g* 808 bleaching appeared to be independent of the intensity. Nevertheless, a more thorough investigation of this point might be useful. The 500 ps decay component is somewhat smaller; it has not been observed before but agrees surprisingly well with the estimate of Kleinherenbrink et al. (*3*) based on the low-temperature yield of charge separation. This very slow phase is accompanied by the formation of a significant amount of $P798^+$. The mechanism of this charge separation step is not understood; a charge separation with BChl *g* 808 as primary reactant has been mentioned as an alternative for "uphill" energy transfer (*4*). In experiments such as those of Figure 2, the amount of $P798^+$ produced via excited BChl *g* 808 is clearly not negligible. Nevertheless the overall efficiency of charge separation may be fairly low, since the 60 ps decay seems to represent mainly a loss channel, as discussed above. As was already noted by Liebl et al. (*26*), this is not easily reconciled with the results of Kleinherenbrink et al. (*3*), who obtained the same efficiency for microsecond flashes at 793 and 808 nm in producing the charge separation at low temperature. The reason for this discrepancy is not clear at present.

A third point concerns the position of the BChl *g* 808 bleaching band with time. The relatively narrow bandwidth of 7 nm of the initial signal upon excitation at 811 nm implies that the maxima of absorption and stimulated emission can only be a few nanometers apart. Subsequent broadening and shifting of the bleaching can be attributed to energy relaxation within the inhomogeneously broadenend BChl *g* 808 system. This relaxation will also contribute to the significant red shift of the steady-state fluorescence spectrum with respect to the maximum of the BChl *g* 808 absorption band (*17*). Our most accurate measurements of the position of the bleaching band are those shown in Figure 4, which were obtained upon excitation at 770 nm. Two distinct time constants were obtained for the red shift of the band, of 2.3 and 120 ps (with 793 nm excitation, time constants of 3.4 and 130 ps were found). Both components represent a shift of the band by about 4 nm, from 806 to 810 nm and from 810 to 813.5 nm. The shift is essentially completed in 400 ps; at that time about a fourth of the amplitude of the bleaching band has not yet decayed, meaning that a significant fraction of the charge separation occurs from the lowest energy form of BChl *g* 808. It is of interest to note that in the early difference spectra the maximum bleaching is at shorter wavelength (806 nm) than the maximum of BChl *g* 808 in the absorption spectrum (809 nm), suggesting that energy transfer from BChl *g* 778 occurs primarily to the short-wavelength forms of BChl *g* 808. The same applies to energy transfer from BChl *g* 793 (Figure 2). This implies that the BChls *g* 808 are not equivalent with regard to position and that their site energies are determined not only by static disorder but also by local differences in binding energies. A similar reasoning may explain the different time constants for the decay and the shift of the bleaching band of 7 and 2.3 ps, respectively.

The overal yield of charge separation cannot be determined directly from our measurements. However, a rough comparison may be made of the yields at 275 K and at 10 K by comparing the absorbance changes due to the formation of excited states and those due to $P798^+$ at both temperatures. When we divide the areas of the bleachings caused by $P798^+$ formed at about 1 ns by those of the maximum bleachings due to excited BChl *g* (measured at roughly 2 ps), we obtain a number of 0.23 at 10 K and of 0.26 at 275 K, both with 770 nm excitation. Although there are obvious uncertainties in this comparison, this would suggest that the yield of charge separation at low temperature is not much less than at 275 K. Nevertheless, the factor of about one-fourth by itself is hard to explain, considering the high differential extinction coefficient of P798 oxidation (32, 33).

Another point concerns the early spectra with 770 nm excitation. Our earliest spectra show bleaching bands near 775 nm, both at 10 and at 275 K, whereas those of Liebl et al. (*22*, *26*), measured with higher time resolution, showed bleachings near 785 (20 K) and 790 nm (300 K). The authors could not offer a plausible explanation for this large red shift with respect to the excitation wavelength, but now it seems that the main reason was in the width of the excitation band.

Of particular interest are the results obtained with excitation at 668 nm. Excitation of Chl *a* 670 resulted in the generation of a significantly larger amount of $P798^+$ relative to the signal of excited BChl *g*, both at 10 K and at 275 K, than did excitation of BChl *g*. At both temperatures the amount of $P798^+$ produced relative to the number of excited states generated in BChl *g* was about 50% larger upon excitation of Chl *a* 670. This implies that a direct pathway for charge separation must exist, operating from excited Chl *a* 670 without involving the excited state of BChl *g*. The effect is somewhat smaller than the corresponding one observed with the reaction center core complex of *Ptc. aestuarii* (*7*), but still it is surprisingly high if one takes into account that a significant part of the absorption at 668 nm may be due to BChl *g*. The yield of this process appeared to be independent of temperature and we may assume that the direct charge separation occurs to the same extent and by the same mechanism at all temperatures.

Two possible schemes for a charge separation upon excitation of Chl *a* 670 not involving the excited BChl *g* antenna pigments were suggested in the previous section. The first one involves direct transfer of excitation energy from Chl *a* 670 to P798 with subsequent charge separation from excited P798. This mechanism can only work if energy transfer from excited P798 to the antenna does not occur: the so-called transfer-to-trap-limited model for charge separation (*2*, *34*) should apply. In contrast to this, in the trap-limited model P798* is in thermal equilibrium with excited

antenna BChl *g* and therefore the excitations would diffuse back into the antenna. This means that there would be no distinction between BChl *g* excited directly or via Chl *a* 670 and P798; in other words, the yield of $P798^+$ eventually produced would be the same for both types of excitation.

The second scheme involves charge separation from excited Chl *a* 670, e.g., by the reaction P798 Chl *a* 670* → $P798^+$ Chl *a* 670^-. This means that Chl *a* 670, which is normally the primary electron acceptor (A_0), would now in the excited state function as initiator of the photochemical reaction. This mechanism will work both for the trap-limited and for the transfer-to-trap-limited model for charge separation. In earlier experiments with reaction center core complexes of green sulfur bacteria, both schemes were compatible with our experimental results, since it was not possible to discriminate whether charge separation was trap-limited or transfer-to-trap-limited (*7*). However, for heliobacteria we concluded that the trap-limited model for charge separation, or a model approaching it, applies at 275 K. This brings us to the conclusion that there is an alternative pathway for charge separation from excited Chl *a* 670 that operates by the second scheme mentioned above, i.e., directly from excited Chl *a* 670. A similar scheme, but starting from accessory BChl (B_A), has been proposed for purple bacteria (*6*).

ACKNOWLEDGMENT

We thank A. H. M. de Wit for growing the bacterial cultures.

REFERENCES

1. Borisov, A. Y. (1990) *Photosynth. Res. 23*, 283−289.
2. Otte, S. C. M., Kleinherenbrink, F. A. M., and Amesz, J. (1993) *Biochim. Biophys. Acta 1143*, 84−90.
3. Kleinherenbrink, F. A. M., Deinum, G., Otte, S. C. M., Hoff, A. J., and Amesz, J. (1992) *Biochim. Biophys. Acta 1099*, 175−181.
4. Chiou, H.-C., Lin, S., and Blankenship, R. E. (1997) *J. Phys. Chem. 101*, 4136−4141.
5. Fischer, S. F., and Scherer, P. O. J. (1987) *Chem. Phys. 115*, 151−158.
6. van Brederode, M. E., Jones, M. R., van Mourik, F., van Stokkum, I. H. M., and van Grondelle, R. (1997) *Biochemistry 36*, 6855−6861.
7. Neerken, S., Schmidt, K. A., Aartsma, T. J., and Amesz, J. (1999) *Biochemistry 38*, 13216−13222.
8. Neerken, S., Permentier, H. P., Francke, C., Aartsma, T. J., and Amesz, J. (1998) *Biochemistry 37*, 10792−10797.
9. Griesbeck, C., Hager-Braun, C., Rogl, H., and Hauska, G. (1998) *Biochim. Biophys. Acta 1365*, 285−293.
10. Kobayashi, M., Oh-oka, H., Akutsu, S., Akiyama, M., Tominaga, K., Kise, H., Nishida, F., Watanabe, T., Amesz, J., Koizumi, M., Ishida, N., and Kano, H. (1999) *Photosynth. Res.* (in press).
11. Amesz, J. (1995) in *Anoxygenic Photosynthetic Bacteria*, (Blankenship, R. E., Madigan, M. T., and Bauer, C. E., Ed.) pp 687−697, Kluwer Academic Publishers, Dordrecht, The Netherlands.
12. Amesz, J. (1995) *J. Photochem. Photobiol.: B, Biol. 30*, 89−96.
13. van de Meent, E. J., Kleinherenbrink, F. A. M., and Amesz, J. (1990) *Biochim. Biophys. Acta 1015*, 223−230.
14. Trost, J. T., and Blankenship, R. E. (1989) *Biochemistry 28*, 9898−9904.
15. van de Meent, E. J., Kobayashi, M., Erkelens, C., van Veelen, P. A., and Amesz, J. (1991) *Biochim. Biophys. Acta 1058*, 356−362.
16. Brockmann H., and Lipinski, A. (1983) *Arch. Microbiol. 136*, 17−19.
17. van Dorssen, R. J., Vasmel, H., and Amesz, J. (1985) *Biochim. Biophys. Acta 809*, 199−203.
18. Kobayashi, M., van de Meent, E. J., Erkelens, C., Amesz, J., Ikegami, I., and Watanabe, T. (1999) *Biochim. Biophys. Acta 1057*, 89−96.
19. Nuijs, A. M, van Dorssen, R. J., Duysens, L. N. M., and Amesz, J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6865−6868.
20. van Noort, P. I., Gormin, D. A., Aartsma, T. J., and Amesz, J. (1992) *Biochim. Biophys. Acta 1140*, 15−21.
21. Lin, S., Chiou, H.-C., Kleinherenbrink, F. A. M., and Blankenship, R. E. (1994) *Biophys. J. 66*, 437−445.
22. Liebl, U., Lambry, J.-C., Leibl, W., Breton, J., Martin, J.-L., and Vos, M. H. (1996) *Biochemistry 35*, 9925−9934.
23. van Kan, P. J. M., Aartsma, T. J., and Amesz, J. (1989) *Photosynth. Res. 22*, 61−68.
24. Kleinherenbrink, F. A. M., Aartsma, T. J., and Amesz, J. (1991) *Biochim. Biophys. Acta 1057*, 346−352.
25. Lin, S., Kleinherenbrink, F. A. M., Chiou, H.-C., and Blankenship, R. E. (1994) *Biophys. J. 67*, 2479−2489.
26. Liebl, U., Lambry, J.-C., Breton, J., Martin, J.-L., and Vos, M. H. (1997) *Biochemistry 36*, 5912−5920.
27. Francke, C., Otte, S. C. M., van der Heiden, J. C., and Amesz, J. (1994) *Biochim. Biophys. Acta 1186*, 75−80.
28. Kennis, J. T. M. (1997) Doctoral Thesis, Leiden University, Leiden, The Netherlands.
29. Smit, H. W. J., van Dorssen, R. J., and Amesz, J. (1989) *Biochim. Biophys. Acta 973*, 212−219.
30. van Kan, P. J. M. (1991) Doctoral Thesis, Leiden University, Leiden, The Netherlands.
31. Breton, J., Martin, J.-L., Fleming, G. R., and Lambry, J. C. (1988) *Biochemistry 27*, 8276−8284.
32. Kleinherenbrink, F. A. M., and Amesz, J. (1993) *Biochim. Biophys. Acta 1143*, 77−83.
33. Kleinherenbrink, F. A. M., Chiou, H.-C., LoBrutto, R., and Blankenship, R. E. (1994) *Photosynth. Res. 41*, 115−123.
34. Timpmann, K., Freiberg, A., and Sundström, V. (1995) *Chem. Phys. 194*, 275−283.

BI992433O