Excited States and Trapping in Reaction Center Complexes of the Green Sulfur Bacterium *Prosthecochloris aestuarii*[†]

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ABSTRACT: The excited states of bacteriochlorophyll (BChl) *a* were studied by pump−probe transient absorption spectroscopy in reaction center core (RCC), Fenna-Matthews-Olson (FMO) and FMO−RCC complexes of the green sulfur bacterium *Prosthecochloris aestuarii*. Excitation at 790 or 835 nm resulted in rapid equilibration of the energy between the BChl *a* molecules of the RCC complex: within 1 ps, most of the excitations had relaxed to the lowest energy level (835 nm), as a result of strong interactions between the BChls. Excitation of chlorophyll *a* 670 resulted in energy transfer to BChl *a* with a time constant of 1.2 ps, followed by thermal equilibration. Independent of the wavelength of excitation, the decay at 835 nm could be fitted with a time constant of about 25 ps, comparable to the 30 ps measured earlier with membrane fragments, which is ascribed to trapping in the reaction centers. Similar results were obtained with the FMO−RCC complex upon excitation at 835 or 670 nm, but the results upon 790 nm excitation were quite different. Again an equilibrium was rapidly reached, but now most of the excitations remained within the FMO complex, with a maximum bleaching at 813 nm, the same as observed in the isolated FMO. Even after 100 ps there was no bleaching at 835 nm and no evidence for charge separation. We conclude that there is no equilibration of the energy between the FMO and the RCC complex and that the efficiency of energy transfer from FMO to the reaction center core is low.

The primary processes of trapping and electron transfer in photosynthetic bacteria have been studied extensively by means of ultrafast time-resolved spectroscopy. This applies in particular to purple bacteria. Green sulfur bacteria have not been studied to such an extent. The main reasons are that, in contrast to the reaction center of purple bacteria, the reaction center core complex of green sulfur bacteria is difficult to isolate in a photochemically active form and, moreover, contains a considerable number of antenna pigments, which makes a spectroscopic study more complicated (*1*).

With respect to their photochemical characteristics, the green sulfur bacteria, like the heliobacteria, resemble photosystem I of green plants. However, they have a much larger and also much more complicated antenna than most other photosynthetic organisms (*2*). The major component, the chlorosome, is located outside the membrane. A second component of the antenna system is the Fenna-Matthews-Olson (FMO)[1] protein complex, which connects the chlorosomes to the membrane. Only the reaction center core (RCC) complex is an intrinsic membrane protein complex. Whereas the main pigment of the chlorosome is either bacteriochlorophyll (BChl) *c*, *d*, or *e*, the FMO and RCC complexes contain BChl *a* (*2*). The primary electron donor, P840, is a BChl *a* dimer (*1*).

Up to now, time-resolved absorption measurements in the picosecond domain have only been performed with membrane fragments, containing the FMO antenna complex in addition to the core complex (*3*−*5*). Among other things, the results indicated that the primary electron acceptor, analogous to A_0 of photosystem I, is a pigment absorbing near 670 nm, later identified as a chlorophyll (Chl) *a* isomer (*6*), Chl *a* 670. The development of a fast and simple method to isolate a purified core-reaction center complex in a photochemically active and relatively stable form (*7*) allowed us to study the excited state and electron-transfer dynamics in more detail than has been possible so far. The present study involves measurements of time-resolved absorbance changes in the femto- and picosecond time region, applied to RCC, FMO−RCC, and isolated FMO complexes of *Prosthecochloris aestuarii*. The experiments indicate that equilibration of the excitation energy between the BChl *a* molecules of the core complex occurs within about 1 ps. Charge separation occurred with a time constant of about 25 ps. No energy transfer was observed between the FMO and the core complexes.

MATERIALS AND METHODS

Prosthecochloris aestuarii, strain 2K, was grown anaerobically in a mixed culture as described by Holt et al. (*8*). RCC complexes and 3FMO−RCC complexes were prepared according to Francke et al. (*7*). The 3FMO−RCC complex contains, in addition to the core complex, about three FMO trimers per reaction center [recent results may indicate a lower number (*9*)]. The RCC complex is devoid of FMO protein, but it contains the pscA and pscB polypeptides, with approximately 17 BChl *a* molecules/reaction center (*7*). The

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[1] Abbreviations: A_0, primary electron acceptor; BChl, bacteriochlorophyll; Chl, chlorophyll; FMO complex, Fenna-Matthews-Olson complex; P840, primary electron donor; RCC, reaction center core.

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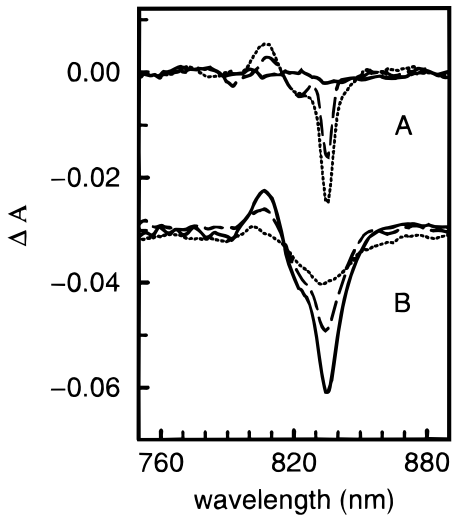

FIGURE 1: (A) Time-resolved difference spectra of the RCC complex upon excitation at 835 nm at 0 ps (solid line), 0.3 ps (dashed line), and 0.4 ps (dotted line) after the onset of the pulse. (B) Spectra at delays 0.6 ps (solid line), 4.6 ps (dashed line), and 85 ps (dotted line). The spectra were plotted with an offset of −0.03 for clarity.

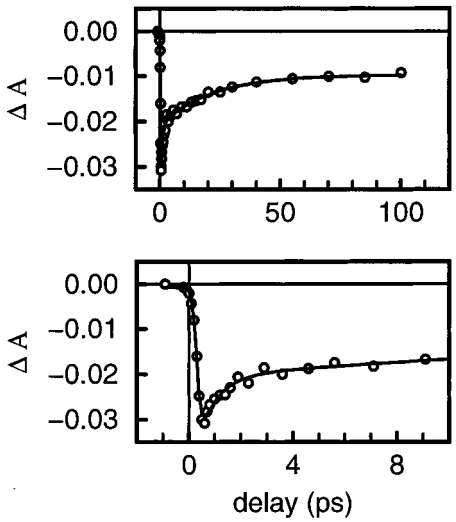

FIGURE 2: Kinetics of absorbance changes at 835 nm upon excitation at 835 nm. Fits were done with two exponential decay constants of 0.8 and 22 ps and a constant value. The relative amplitudes of the 0.8 ps, 22 ps, and the constant components were 1:0.75:0.6.

final preparations were dissolved in a buffer containing 20 mM potassium phosphate (pH 6.5) and 2.5 mM Triton X-100 and had an absorbance of 0.5−0.6/mm at 835 nm, except where otherwise indicated. To all samples, 30 mM sodium ascorbate and 20 μM *N*-methylphenazonium methosulfate (PMS) were added to keep P840 reduced in the dark. The FMO complex was isolated according to Francke and Amesz (*10*) and dissolved in 50 mM Tris/HCl and 200 mM sodium chloride (pH 8.3). Its concentration was adjusted to an absorbance of 1/mm at 809 nm.

Time-resolved transient absorption measurements were performed with a home-built amplified dye laser system with continuum generation and optical multichannel analyzer (OMA) detection, operating at 10 Hz, described by Kennis (*11*). The time resolution was 300 fs. Wavelength-selective excitation pulses were obtained by passing the continuum pulse through an amplifying dye-cell (pumped by a frequency-doubled, Q-switched Nd:YAG laser) and subsequently through a suitable interference filter with a band width of 7−13 nm. LDS 698 was used for amplification around 670 nm and LDS 821 for amplification in the 790−835 nm region. Pump and probe pulses were polarized parallel to each other. In the case of spectrally broad excitation, wavelengths shorter than 780 nm were cut off with an RG780 filter (Schott). At each delay a few thousand spectra were recorded and averaged. We corrected for the group velocity dispersion by applying a third-order polynomial function which was obtained by measuring the dispersion in CS_2 (*12*, *13*). The samples were put in a moving cuvette in order to avoid accumulation of oxidized P840. The optical pathway was 1 mm. All measurements were performed at 2 °C.

RESULTS

RCC Complex. Figure 1 shows time-resolved spectra of the RCC complex upon excitation at 835 nm at different delays of up to 85 ps. A narrow bleaching at 835 nm is observed, together with a smaller one at 822 nm and a positive band around 807 nm. The bleachings are presumably due to ground-state depletion upon formation of singlet-excited BChl *a*, together with stimulated emission, while the positive band at 807 nm may be ascribed to excited state absorption. During the first few hundred femtoseconds, the main bleaching band had approximately the same spectral width as the pump pulse, indicating that the excitations were confined to those BChls that were initially excited. The bleaching then broadened very rapidly, reached a maximum height after 0.6 ps and subsequently decayed to a constant value. The maximum bleaching was 3.8% of the absorbance at 816 nm.

From the time-resolved spectra, the kinetics at 835 nm were derived, shown in Figure 2. The decay of the bleaching could be fitted with time constants of 0.8 ± 0.3 and 22 ± 3 ps and an irreversible component. The time constant of about 22 ps may be ascribed to trapping in the reaction center (*5*). Within the error of the measurement, the excited-state absorption at 807 nm disappeared along with the 22 ps kinetic decay component. The irreversible component may be ascribed to photooxidized P840, which is completely stable on this time scale (*7*). The value for $\Delta A_{835}/A_{816}$ at a delay of 100 ps was 0.013, which is about one-fourth of the number obtained with saturating continuous light (*7*).

Figure 3 shows spectra obtained upon excitation at 790 nm. Since the preparation does not contain any FMO-protein, excitation is again in the BChl *a* of the core at this wavelength. At short delays, the spectra were quite different from those obtained with 835 nm excitation, with a main bleaching near the wavelength of excitation, 790 nm. However, within a few hundred femtoseconds, strong bleaching bands at longer wavelengths developed, and after about one picosecond, the spectrum resembled that obtained upon 835 nm excitation. The kinetics at 835 nm showed an approximately 25 ps decay and an irreversible component, the latter again being ascribed to $P840^+$.

Taken together, our experiments indicate that there is a rapid equilibration of the energy among the excited BChl *a* molecules. Upon 835 nm excitation, the equilibration is visible as a broadening of the bleaching around 835 nm

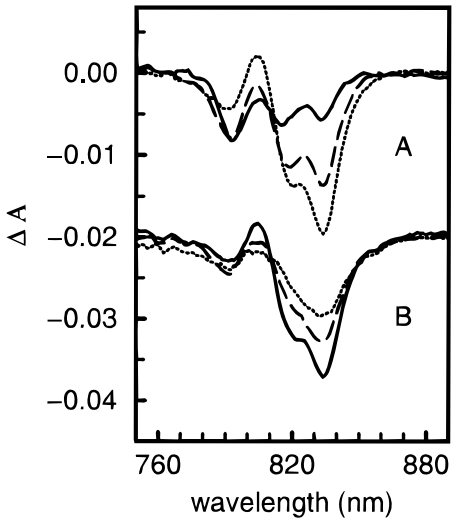

FIGURE 3: (A) Time-resolved spectra of the RCC complex upon excitation at 790 nm at delays 0.3 ps (solid line), 0.5 ps (dashed line), and 0.9 ps (dotted line). (B) Difference spectra at delays 4 ps (solid line), 20 ps (dashed line), and 120 ps (dotted line); offset: −0.02.

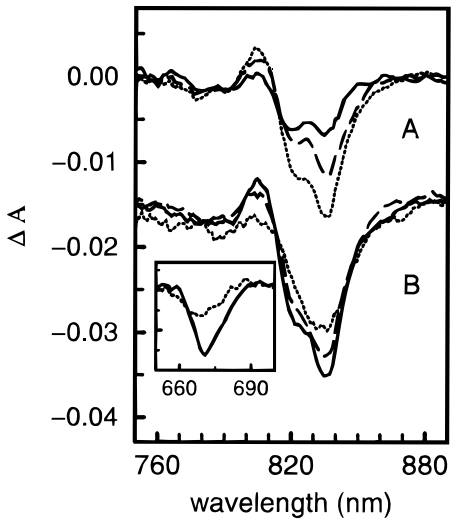

FIGURE 4: (A) Time-resolved spectra of the RCC complex upon excitation at 670 nm at delays 0.6 ps (solid line), 1 ps (dashed line), and 2 ps (dotted line). (B) Difference spectra at delays 7 ps (solid line), 21 ps (dashed line), and 110 ps (dotted line); offset: −0.015. (Inset) 0.5 ps (solid line) and 100 ps (dotted line).

(Figure 1), while excitation at 790 nm is followed by a growth of the bleaching bands at 835 and 815 nm and a red shift of the latter one (Figure 3). The equilibration appears to be largely completed within the first picosecond of excitation. The 0.8 ps kinetic component may be representative of this process, but the transient spectra show that also more rapid components are involved, which are only partly resolved with the time resolution of our apparatus.

Figure 4 shows time-resolved spectra with excitation at 670 nm, with selective excitation of Chl *a* 670. Around 670 nm, a rapid bleaching of the absorption band was observed, part of which decayed with a time constant of 1.2 ps (Figure 5A). The rise time at 835 nm was the same as the decay time at 670 nm, indicating that at least part of the Chls *a* 670 transfer their excitation energy efficiently to BChl *a*. The spectrum measured at an early time after excitation (solid line in Figure 4A) indicates that this transfer occurs about equally to BChls *a* absorbing near 820 and near 835 nm,

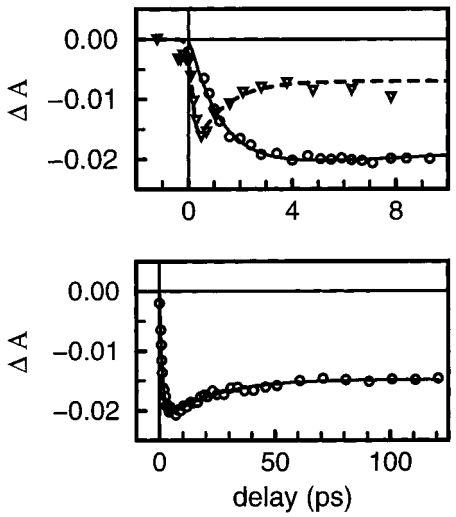

FIGURE 5: Kinetics of absorbance changes at 835 nm upon 670 nm excitation fitted with a rise time of 1.2 ps, a decay time of 25 ps and a constant component (solid lines). The dashed line (triangles) gives the kinetic trace at 671 nm, fitted with a decay of 1.2 ps and a constant component.

after which thermal equilibrium is established. The decay at 835 nm could be fitted with a time constant of 25 ps and an irreversible component, again ascribed to trapping in the reaction center and to formation of $P840^+$.

If we compare the absorption difference spectra for excitation at the different wavelengths, 670, 790, and 835 nm, it is striking that these spectra, especially above 800 nm, are very similar to each other already at a delay of 1−2 ps. The main difference is a persistent bleaching near 790 nm upon excitation at 790 nm, which is absent in the other spectra. This band can probably be ascribed to a long-lived excited state of some free BChl *a*. This is supported by steady-state fluorescence measurements showing an emission band at 800 nm (data not shown), which may be ascribed to BChl *a* molecules which do not transfer their energy to the primary electron donor.

Figure 6 shows time-resolved spectra throughout the entire Q_y region, obtained with broad-band excitation pulses centered at 820 nm. In the BChl *a* region, the spectra and the kinetics were similar, except for the amplitude, to those earlier observed upon narrow-band excitation. A maximum absorbance decrease at 835 nm of 0.14 was reached, which is 16% of the absorbance at 816 nm. Again, a fast decay and a kinetic constant of about 25 ps were observed, together with an irreversible component. From 640 to 750 nm, a broad absorption increase was measured, on which a bleaching at 670 nm was superimposed. A broad positive band in this region was earlier observed for membranes of *P. aestuarii* (*3*, *4*). The bleaching around 670 nm was clearly visible at 9 ps (Figure 6B) and did not decay significantly on the time scale of the measurement. The time-resolved spectrum at a delay of 120 ps in Figure 6B can be interpreted as the difference spectrum of the charge separated state $P840^+A_0^-$. The ratio $\Delta A_{835}/A_{816}$ at a delay of 120 ps in this case was 0.058. This is in good agreement with the value of 0.055 found by us with saturating continuous illumination (*7*).

FMO and 3FMO−RCC Complexes. Time-resolved measurements were also performed on 3FMO−RCC and FMO

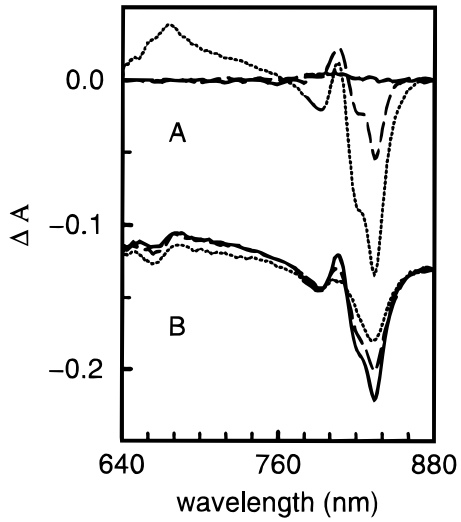

FIGURE 6: (A) Time-resolved spectra of the RCC complex upon excitation with a spectrally broad pulse (fwhm 30 nm) centered at 820 nm, at delays 0 ps (solid line), 0.4 ps (dashed line), and 3.5 ps (dotted line). (B) Difference spectra (offset: −0.13) at delays 9 ps (solid line), 30 ps (dashed line), and 120 ps (dotted line).

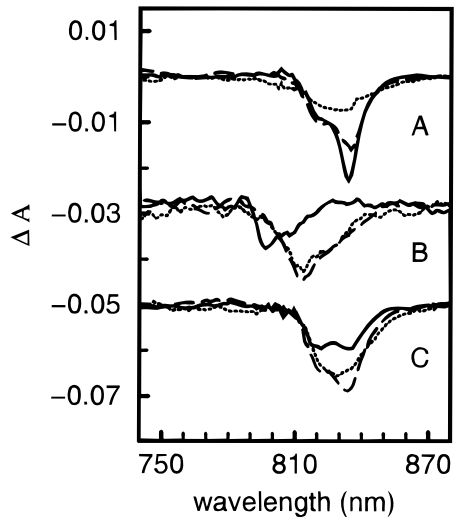

FIGURE 7: Absorbance difference spectra of the 3FMO−RCC complex upon excitation at 835 (A), 790 (B), and 670 nm (C) at delays of 0.4 ps (solid lines), 4 ps (dashed lines), and about 90 ps (dotted lines); offsets: B, −0.03; C, −0.05. The absorbance for measurements with excitation at 790 nm was 1.0/mm at the maximum. The corresponding spectra were enlarged two times.

complexes. Some of the results are shown in Figures 7 and 8. Figure 7 displays absorption difference spectra for the 3FMO−RCC complex at three delays, following excitation at 835, 790, and 670 nm, respectively. Spectra and kinetics obtained upon excitation at 670 and 835 nm were similar to those measured for the RCC complex, as was to be expected, since under these conditions excitation is mainly in the RCC complex. Excitation at 835 nm resulted in a narrow bleaching, which broadened rapidly and decayed with biexponential kinetics, which could be fitted again with time constants of 0.8 and about 25 ps and an irreversible component. Upon 670 nm excitation, the kinetics at 835 nm were fitted with a rise time of 1.2 ps and a decay of about 25 ps to a constant value.

The above observations indicate that the photochemical and photophysical properties of isolated RCC complexes and of those bound to the FMO antenna are the same. Quite

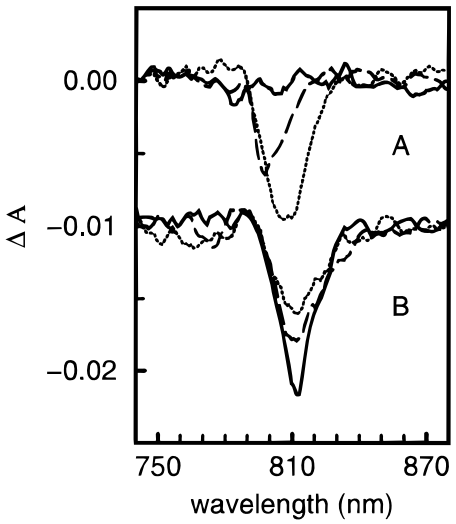

FIGURE 8: Time-resolved spectra of the isolated FMO complex upon excitation at 790 nm at different delays. (A) 0.1 ps (solid line), 0.3 ps (dashed line), and 0.5 ps (dotted line). (B) 1.9 ps (solid line), 20 ps (dashed line) and 95 ps (dotted line); offset: −0.01.

different results, however, were obtained with excitation around 790 nm where the light is absorbed predominantly by the FMO complex. An initial bleaching was formed near 795 nm, which rapidly shifted to 813 nm. This shift was completed within 1 ps, after which the spectrum remained essentially unchanged during the next 100 ps, except for a slow decrease of the amplitude of the bleaching. Even at a delay of 90 ps, no significant bleaching was observed at 835 nm and no evidence for charge separation was obtained. If we compare these results with measurements on the isolated FMO complex (see below), we estimate that at least 80% of the excitations stay within the FMO complex for at least 100 ps.

Figure 8 shows time-resolved spectra measured with isolated FMO complexes. These spectra are very similar to those obtained with the 3FMO−RCC complex upon excitation at 790 nm. Initially, a bleaching is formed at 795 nm, followed by a shift to 812 nm, which is complete after 1 ps.

We conclude that even at a time scale of 100 ps very little energy transfer occurs from the FMO complex to the core. The same conclusion was reached earlier for isolated membranes (*5*, *14*) and for other FMO−RCC preparations (*14*).

DISCUSSION

Our time-resolved measurements show that excitation results in rapid equilibration of the energy between the BChl *a* molecules of the RCC complex. After about one picosecond, an absorbance difference spectrum was obtained which was essentially independent of the wavelength of excitation, with relaxation to the lowest energy level (835 nm). Following a pulse at 835 nm, the excitations were at first confined to those BChls that were initially excited, and the equilibration was mainly reflected by a subsequent broadening of the bleaching band. Upon excitation at 790 nm, the initial bleaching at this wavelength was followed by a shift of the excitation to lower energies. Excitation of Chl *a* 670 gave rise to rapid energy transfer to BChl *a*, followed by equilibration as described above. In all cases, the equilibration was complete in 1−2 ps; a 0.8 ps kinetic

component may be associated with this process, but faster components are clearly also involved.

The difference spectra obtained at a delay of about 1 ps (e.g., the solid line in Figure 1B) are reminiscent of those observed in the LH2 antenna complex of purple bacteria (*15*) and in J-aggregates (*16*), the excited-state absorption band indicating significant exciton interaction in the RCC complex. However, if we consider the bleaching due to the formation of $P840^+$ under the same conditions, it appears that the amplitude of the maximum bleaching in the excited state is about three times that of $P840^+$ (Figure 1B). This number is significantly less than the corresponding one in purple bacteria (*17*), and taking into account that it contains a contribution by stimulated emission, it appears that the effect of exciton interaction on the extinction coefficient is not very large. This may be due, at least in part, to a lack of symmetry, as in the FMO complex (*18*).

Irrespective of the wavelength of excitation, a decay component of about 25 ps was found at 835 nm, which we ascribe to trapping in the reaction center. This result may be compared to the 30 ps time constant measured with isolated membranes by Kramer et al. (*5*). It appears that trapping in green sulfur bacteria occurs with nearly the same time constant as in heliobacteria: 25−30 ps (*19*, *20*). A similar rate of charge separation was also reported for photosystem I (*21*). Our results imply that spectral equilibration is essentially complete before the charge separated state $P840^+A_0^-$ is formed. So far we observed no fast kinetic component for charge separation as reported by Liebl et al. (*22*) for heliobacteria.

The difference spectrum of the "irreversible component", obtained after the charge separation was completed, is much simpler than that of $P840/P840^+$ obtained in the millisecond time range or upon continuous illumination (*7*). This was noted already by van Bochove et al. (*23*) and may be explained by the absence of Stark effects on neighboring pigments when the separated charges are still close together.

The absorbance changes at 670 nm are still not well understood quantitatively. A bleaching at this wavelength was first observed by van Bochove et al. (*23*) and attributed to reduction of the primary electron acceptor A_0. In Figure 6, the amplitude of the bleaching at 670 nm is only one-fourth of that due to P840 oxidation (measured at 100 ps after the pulse). This is significantely less than expected, taking into account that the extinction coefficients of Chl *a* 670 [95 mM^{-1} cm^{-1} (*9*)] and P840 are probably roughly the same. It seems unlikely that there is sufficient absorption by the reduced acceptor to explain this effect (*24*). The bleachings observed by Nuijs et al. (*3*), and by us upon excitation at 670 nm (Figure 4, measured at a delay of 110 ps) were somewhat larger than those upon excitation around 820 nm (Figure 6), but here, some contribution by excited Chl *a* 670 cannot be excluded. The initial bleaching induced by the pulse at 670 nm, measured at 0.5 ps, is about the same as that of $P840^+$, but this must be ascribed to excited Chl *a* 670.

The spectral dynamics of the isolated FMO complex indicate, as in the RCC complex, a rapid thermal equilibration of the excited-state energies. A more detailed study of this phenomenon at room temperature in the FMO complex from *Chlorobium tepidum* was made by Savikhin and Struve (*25*). The "final" difference spectrum, reached within about 1 ps, shows a maximum at 812 nm (*5*, *26*).

The conclusions drawn from the measurements on the 3FMO−RCC complex upon excitation at 670 and 835 nm are in principle the same as for the RCC complex. However, even at 100 ps after the pulse, the absorbance difference spectrum obtained with 790 nm excitation is quite different and closely resembles that obtained with isolated FMO complexes. We conclude that there is no equilibration of the energy between the FMO protein and the RCC complex and that the excitations stay within the FMO complex on the time scale of our measurements. Recent results indicate that in solubilized FMO−RCC complexes part of the FMO protein may be loosely and nonspecifically bound (*9*). This may, at least in part, explain the absence of substantial energy transfer from FMO protein to the RCC complex in these and earlier (*14*) measurements, but it does not explain why the same result was obtained with isolated membranes (*5*, *14*).

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