Excitation Wavelength Dependent Spectral Evolution in *Rhodobacter sphaeroides* R-26 Reaction Centers at Low Temperatures: The Q_y Transition Region

Su Lin, Jon Jackson, Aileen K. W. Taguchi, and Neal W. Woodbury*

Department of Chemistry and Biochemistry and the Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287-1604

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The spectral evolution associated with energy and electron transfer in quinone-depleted reaction centers from *Rhodobacter sphaeroides* strain R-26 was investigated at low temperatures using femtosecond transient absorbance spectroscopy as a function of excitation wavelength. Laser pulses of 150 fs duration and 5 nm spectral bandwidth at 760, 800, 810, and 880 nm were used to selectively excite the 760 nm transitions of the bacteriopheophytins (H), the bacteriochlorophyll monomer (B) transitions near 800 and 808 nm, and the 880 nm bacteriochlorophyll dimer (P) transition (810 nm excitation also presumably excites the upper exciton band of P). While the general features of the kinetic and spectral behavior observed are similar to previous room-temperature measurements, the excitation wavelength dependence is generally more pronounced and much longer-lived. The absorbance changes throughout the 740−1000 nm region are excitation wavelength dependent. These differences are clearly evident after several tens of picoseconds, and some spectral differences persist for hundreds of picoseconds. Previous reports have explained much of the excitation wavelength dependence of reaction centers in terms of formation of charge separation intermediates directly from B* or H* such as P^+B^- or B^+H^-. However, it is unlikely that either of these charge-separated states would persist after several tens or hundreds of picoseconds. Though this certainly does not rule out charge separation directly from excited states of B and H, it suggests that other explanations must be put forth to account for at least a large fraction of the excitation wavelength dependence observed. A likely possibility is spectral heterogeneity within the reaction center population, resulting in optical selection by different excitation wavelengths. This could explain much of the excitation wavelength dependent spectral evolution on time scales longer than 1 ps.

Introduction

Light-driven electron-transfer reactions in purple bacteria occur in the reaction center and are initiated when the special pair (P, a pair of bacteriochlorophyll molecules), located near the periplasmic side of the membrane, absorbs a photon forming a singlet excited state (P*). Electron transfer then occurs through a series of protein-bound cofactors resulting in a transmembrane charge-separated state.[1−7] Extensive spectroscopic studies on the femtosecond and picosecond time scales have shown that initial electron transfer occurs from the lowest excited state of P, P*, to the A-branch bacteriopheophytin, H_A, within 3 ps.[1−3] The reaction center structure has a near 2-fold symmetry, resulting in two potential electron-transfer chains, but only the A-branch is significantly active in electron transfer (the active branch is designated A and the inactive branch, B). The mechanistic description of the initial charge separation event is not entirely clear, but $P^+B_A^-$ plays a role in the electron transfer between P and H_A.

There is little temperature dependence to the initial electron-transfer rate which speeds up by only about a factor of 2 at low temperatures (10−20 K). The rate is also rather insensitive to driving force; electron transfer occurs on the picosecond time scale at low temperature even in mutants with P/P^+ midpoint potentials more than 100 mV above that of wild type.[8,9] The insensitivity of the charge separation rate to the driving force and temperature as well as the apparently incomplete vibrational relaxation on this time scale[10−15] challenges the conventional notion that the early energy- and electron-transfer reactions occur between weakly coupled, vibrationally relaxed states.

In systems where either vibrational relaxation is incomplete or coupling is strong, one might expect that the spectra and kinetics of absorbance changes on the time scale of the early (subpicosecond to a few picoseconds) energy- and electron-transfer reactions would be excitation wavelength dependent. In previous studies, purple bacterial reaction centers were excited in different transition bands at both room temperature and low temperatures.[16−24] Excitation energy transfer from H and/or B to P has been demonstrated to occur on the 100 fs time scale by monitoring the kinetics of P band bleaching at room temperature[16,17] or 10 K,[25] by measuring the decay lifetime of the B* absorbance decrease at room temperature,[20] by measuring the rise time and induced anisotropy of the spontaneous emission from P using fluorescence up-conversion techniques at 85 K,[21] by measuring the kinetics of several near- and mid-infrared transitions using polarized light at room temperature,[22] and by monitoring the kinetics of anisotropy using pump−probe techniques in the B band wavelength region.[23] It appears likely that this transfer is through the upper excitonic excited singlet state of P. The transfer from H to P is about 50% slower than that directly from B and probably passes through B. Most of the reports that have considered the question in detail have

* To whom correspondence should be addressed. E-mail Nwoodbury@asu.edu.

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suggested that energy transfer in this system is too rapid to be adequately described by a Förster-type weak coupling model,[26] though this is not universally accepted.[22,27]

In addition to the kinetic measurements described above, the excitation wavelength dependence of transient absorbance spectra as a function of time have also been measured, and additional spectroscopic features have been identified and investigated.[24,28] Absorbance change spectra as a function of time were measured simultaneously from 730 to 970 nm at room temperature with different excitation wavelengths. The results illustrated that the spectral evolution is much more complicated than one would have expected for energy and electron transfer between vibrationally relaxed, weakly coupled states. The time-dependent spectra were excitation wavelength dependent for many picoseconds after excitation. Excitation of the monomer bacteriochlorophylls at 800 nm or the bacteriopheophytins at 760 nm resulted in apparently less stimulated emission from P* than is seen upon 860 nm excitation of the special pair. Additionally, there is more ground-state bleaching in the 760 and 800 nm region observed upon direct excitation of the monomer bacteriochlorophylls or bacteriopheophytins. This suggested the possibility that charge separation might occur from B* or H* either directly or with less involvement of P*.[24] More recently, Van Brederode et al. have analyzed the possibility of direct charge separation from B* and H* in more detail using a mutant with a slower rate of electron transfer.[29,30] They concluded from both steady-state excitation spectra and femtosecond transient absorption measurements that, in the mutant YW(M210), excitation of B resulted in very fast charge separation from B* forming P^+B^- and B^+H^-. Vos et al.[28] have looked at R-26 reaction centers at low temperature using excitation into both H and B and observed large, long-lived excitation wavelength dependence across the near-infrared spectrum. They also attributed much of the excitation wavelength dependence to direct charge separation from B* or H* (particularly from H*).

To investigate the possibility of alternate charge separation pathways from B* and H* more thoroughly, we have performed detailed excitation wavelength dependence studies at 12 K. In particular, we have investigated the long-time excitation wavelength dependence of spectral changes at this temperature, considering whether these changes are consistent with a mechanism involving direct charge separation upon excitation of B or H.

Materials and Methods

Procedures for the preparation of *Rb. sphaeroides* R-26 reaction centers were described previously.[31] Both quinones were removed by treatment with 4% *N,N*-dimethyldodecylamine-*N*-oxide (LDAO) and *o*-phenanthroline as described previously.[32,33] Low-temperature measurements were performed using a closed circulated helium displex (APD). Samples were prepared in 67% (v/v) glycerol and 33% of a reaction center solution containing ($TLE^{0.025\%}$) 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), and 0.025% LDAO. The optical density at low temperature and 800 nm was typically 1.6 in the low-temperature sample cell used for these studies which had an optical path length of 1.2 mm.

The femtosecond transient absorption spectrometer was described previously.[8,24] Excitation pulses have a time duration of 150−200 fs and spectral width of 5 nm at 540 Hz repetition rate. The spectral dispersion correction and data analysis have also been described.[15,24]

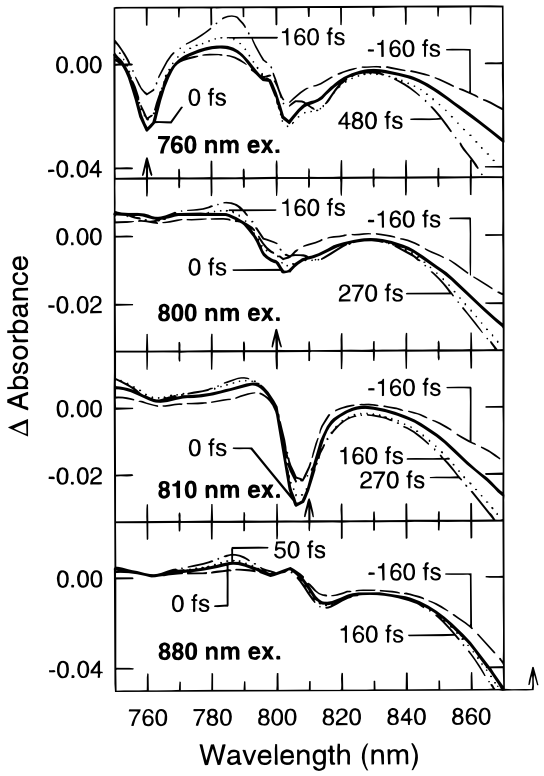

Figure 1. Time-resolved spectra of R-26 at 12 K with excitation at 760, 800, 810, and 880 nm at early times. Arrows in each panel indicate excitation wavelength. Excitation pulse widths are 150 fs in time and 5 nm in spectrum.

Results

Early Time Spectral Differences. Time-resolved spectra from quinone-removed reaction centers of R-26 were recorded over a 5 ps time scale at 54 fs time intervals and a temperature of 12 K. The time-resolved spectra were measured in the wavelength region from 700 to 1000 nm using 760, 800, 810, and 880 nm excitation pulses. Excitation at 760 nm should result in predominantly a mixture of bacteriopheophytin excited states (H_A* and H_B*), 800 nm excitation should result in a fairly even mixture of A-side and B-side monomer bacteriochlorophyll excited states, and 810 nm excitation also results in a mixture of monomer bacteriochlorophyll excited states, but probably favors B_B* formation.[34,35] Excitation at 810 nm also presumably excites the upper exciton band of P.[20,23,28] Spectra were recorded using probe pulses polarized at the magic angle with respect to the excitation pulses. The excitation pulse intensity was adjusted such that less than 15% of the reaction centers were excited per pulse. This corresponded to an approximate pulse energy of 3−5 μJ in a 1−2 mm diameter spot size. Time zero is defined as the time when the bleaching at the excitation wavelength reaches its maximum. In some data sets the time of maximum bleaching is somewhat uncertain due to the complexity of spectral shifting and broadening. Because of this, there is a roughly 50 fs uncertainty in the zero position. Detailed spectral changes from 750 to 870 nm within the time duration of the excitation pulse are compared in Figure 1. In each panel, the 0 ps spectrum is plotted as a bold solid line.

Excitation at 760 nm results initially in an absorbance decrease at 760 nm which grows in during the duration of the pulse (from −160 to 0 fs), maintaining a constant band shape and peak position. The 0 ps spectrum shows main bleaching bands at 760, 802, and 880 nm as well as shoulders at 796 and 814 nm. Bleachings at 802 and 814 nm are not fully developed

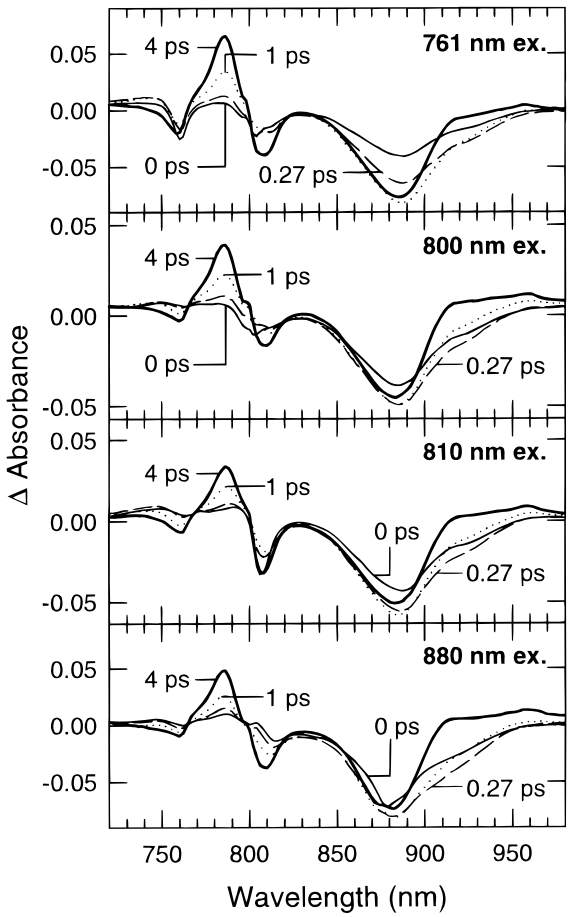

Figure 2. Time evolution at 12 K of R-26 reaction center absorbance change spectra within the first 4 ps after laser excitation at 761, 800, 810, and 880 nm.

until 160 fs. The apparent absorbance decrease due to stimulated emission at 920 nm reaches its maximum at about 480 fs (data not shown) while the bleaching of the H band and the B band have partly decayed. This early time spectral evolution is most likely due to the energy transfer from H_A to other cofactors and excited-state relaxation. The energy-transfer processes from H to B and P are similar to those observed at room temperature, except that detailed band structures in the 800 nm region are well-resolved at 12 K. The 796 and 802 nm bands are likely due to the monomer bacteriochlorophyll transitions on the active and inactive branch.[34,35] The bleaching around 814 nm may originate, at least in part, from the upper excitonic transition of P.[20,23,28]

In the case of 800 nm excitation, the absorbance initially decreases at both 802 and 798 nm (Figure 1). The 798 nm component of the absorbance decrease is much more pronounced than is seen with 760 nm excitation, which may be due at least in part to a different ratio of B_A* to B_B* at early times using these two excitation wavelengths. Using 800 nm excitation, a broad absorbance increase appears in the 750−780 nm region upon excitation, and only a very weak superimposed bleaching at 765 nm is observed. This is in contrast with the initial absorbance changes observed using 760 nm excitation which include simultaneous bleachings of the H and B bands. Less than 5% of the final absorbance decrease due to charge separation is observed in the 760 nm region initially when excitation is at 800 nm (Figures 1 and 2). Bleachings at 814 nm (probably the upper exciton transition of P) and 880 nm (the lower exciton transition of P) grow in as the 796 and 802 nm absorbance changes (mostly B_A and B_B) decay (Figure 1). The stimulated emission from P reaches its maximum 270 fs after excitation at 800 nm.

Excitation at 810 nm initially induces major bleachings at 806 and 880 nm (Figure 1). The 806 nm band broadens toward the longer wavelength side during the first few hundred femtoseconds, and the absorbance decrease at 880 nm becomes larger during the same period. As with 800 nm excitation, the stimulated emission reaches its maximum within 270 fs.

Figure 1 also shows the absorbance changes from 750 to 870 nm between −160 to 160 fs with excitation at 880 nm. The 880 nm excitation induces a broad absorbance increase at wavelengths below 800 nm with smaller features near 765 and 790 nm. Absorption decreases at 798 and 814 nm are also observed at early times. Though significant, changes at wavelengths shorter that 800 nm are small (<10%) compared to the absorbance changes at the wavelength of excitation (Figure 2). The stimulated emission at 920 nm is fully developed within 50 fs with 880 nm excitation (not shown). In contrast to other excitation wavelengths studied, there is no evidence for a substantial absorbance decrease at 802 nm during the first few hundred femtoseconds when 880 nm excitation is used.

Comparing the results of Figure 1 with excitation wavelength dependent data measured at room temperature,[24] spectra recorded at 12 K show several resolved absorbance change bands in the 800 nm region. In general, one sees larger differences between the absorbance change spectra using different excitation wavelengths at 12 K than at room temperature. This is in part due simply to the fact that the absorbance bands are more resolved, so small changes are more localized in the spectrum and have a greater maximum optical density change. However, it is clear that on long time scales (tens to hundreds of picoseconds, see below) there is substantially more excitation wavelength dependence to the difference spectra at low temperature compared to room temperature, because at room temperature the difference spectra taken at different excitation wavelengths converge after tens of picoseconds. As discussed later, this may be due to less interconversion between reaction center conformations at low temperature on the picosecond time scale.

Spectral Evolution on the Picosecond Time Scale. Figure 2 shows the spectral evolution from 720 to 980 nm on the picosecond time scale using excitation at 761, 800, 810, and 880 nm. Though the spectra are more similar using different excitation wavelengths on the several picosecond time scale than they were on the femtosecond time scale, detailed spectral differences persist even after 4 ps (solid bold lines, Figure 2). The H band bleaching is larger and narrower under 760 nm excitation. A narrower band at 808 nm with a larger amplitude (relative to the maximum bleaching in the P band) is observed using 810 nm excitation compared to a broader, weaker absorbance decrease centered at 810−812 nm using other excitation wavelengths.

Figure 2 also shows differences in the 920 nm region. As seen previously by these authors and by others, there appears to be significantly less stimulated emission at early times when excitation at 800 or 760 nm is used compared to 880 nm.[24,28−30] This can be seen by comparing the difference between the 0.27 ps P* spectrum and the 4 ps P^+ spectrum in each panel of Figure 2 at the different excitation wavelengths.

Excitation Wavelength Dependent Kinetics. Kinetic traces at several probe wavelengths using 760, 800, and 810 nm excitation are shown in Figure 3. Data were recorded on a 5 ps time scale at 54 fs per point. In each panel, kinetics at the

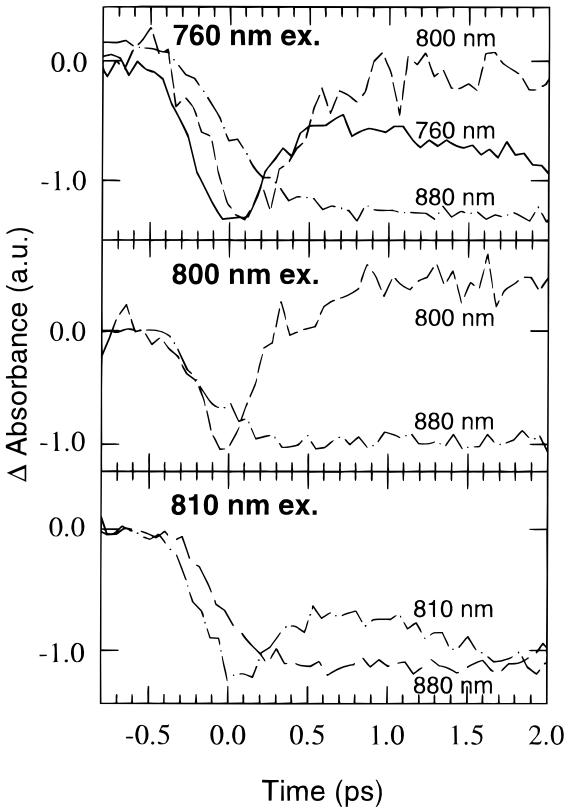

Figure 3. Absorbance change kinetics of R-26 reaction centers at 12 K for several probe wavelengths using excitation at 760, 800, and 810 nm. Data were recorded on a 5 ps time scale with a time resolution of 54 fs per point. Kinetic traces in each panel are normalized to their maxima for ease of comparison.

excitation wavelength and at 800 and 880 nm are plotted after normalization at their bleaching maxima. The estimates of energy-transfer times given below are based on time shifts in the half-maximal bleaching of the different absorbance bands. It was not possible to accurately fit the energy-transfer kinetics with exponential functions across the whole wavelength region measured for two reasons. First, many of the absorbance changes occurring at early time are not simple, wavelength independent exponential decays and rises. They come about from shifting and broadening absorbance bands or coherent effects and cannot be fit by normal global exponential decay analysis. Second, the excitation and probe pulses have durations similar to the lifetimes of the excited states necessitating deconvolution of the excitation and probe pulse widths in the fitting. It is difficult to generate an accurate representation of these pulse widths, and the rise times are quite sensitive to this.

Using 760 nm excitation, delays of roughly 100 and 250 fs are seen in the kinetics of bleaching at 800 and 880 nm, respectively. The bleaching at 880 nm using 800 nm excitation is delayed by less than 100 fs (though there appears to be a slower part to the 880 nm rise as well), a significantly shorter delay than that observed using 760 nm excitation. A somewhat longer delayed rise of 150 fs at 880 nm is observed using 810 nm excitation. Excitation dependent kinetics at each transition wavelength again reflect the processes of energy transfer and vibrational relaxation among cofactors in reaction centers. These characteristics are similar to what has been observed previously.[20−25,27]

Spectral Changes on the 50 ps Time Scale. Absorbance change spectra with 760, 800, and 880 nm excitation were also recorded on the 50 ps time scale (100 spectra were taken at 0.5 ps intervals). Figure 4 compares the time-resolved spectra at 35 ± 5 ps as a function of excitation wavelength. Spectra using different excitation conditions are normalized at the peak of the absorbance decrease in the 880 nm region. (Though the position of normalization is somewhat arbitrary, the spectral shape changes described below are largely independent of the normalization.) The dominant initial energy- and electron-transfer processes at low temperature are complete within picoseconds. Though small-amplitude spectral changes occur on the 10 ps time scale (see below), even these processes are essentially static 35 ps after the laser excitation. (Note that secondary electron transfer is blocked by quinone removal.) However, Figure 4 shows that significant differences still exist in all major bands 35 ps after the excitation. Excitation at 760 nm results in a larger bleaching at 760 and 802 nm than is observed with other excitation wavelengths. Excitation at 880 nm results in a smaller absorption increase near 780 nm compared with other excitation wavelengths. Note that these measurements have been extended out to 200 ps using 800 and 760 nm excitation, and the excitation wavelength dependence of the spectral shapes present at 35 ps is at least partially present at 200 ps (particularly the increased bleaching near 760 nm when 760 nm excitation is used). However, due to baseline variation on this longer time scale, the more subtle features are harder to compare between excitation wavelengths (data not shown). Figure 4 also shows a wavelength dependent maximum of the bleaching in the 880 nm region due to P ground-state depletion. These measurements were repeated numerous times, and while the excitation wavelength dependence in the 760 and 800 nm regions was reproducible, the exact nature of the excitation wavelength dependence of absorbance changes between 830 and 920 nm varied from experiment to experiment. For this reason, the excitation wavelength dependence of long time absorbance changes in the 830−920 nm region will not be discussed further except to say that this appears to be very dependent on exactly how the sample is prepared and cooled. Long-lived broadening and shifting of P's ground-state absorbance band bleaching as a function of excitation wavelength *within* the P band has been observed previously by Peloquin et al. using excitation wavelengths between 840 and 910 nm and was attributed largely to heterogeneous broadening.[14,15]

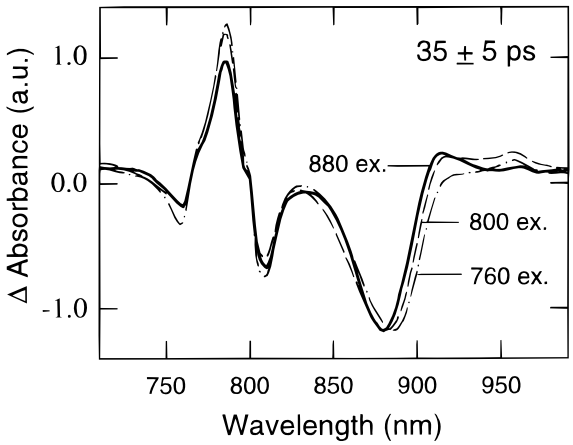

Figure 4. Time-resolved absorbance change spectra of R-26 reaction centers recorded at 35 ± 5 ps with excitation at 760, 800, and 880 nm. Spectral resolution is 2 nm and time resolution is 0.5 ps per point. Spectra were recorded at 12 K and normalized at the wavelength of greatest P band bleaching near 880 nm.

Excitation wavelength dependent differences can also be observed in the dynamics of the spectral evolution on the 1−40 ps time scale. Figure 5a shows time-resolved spectra recorded

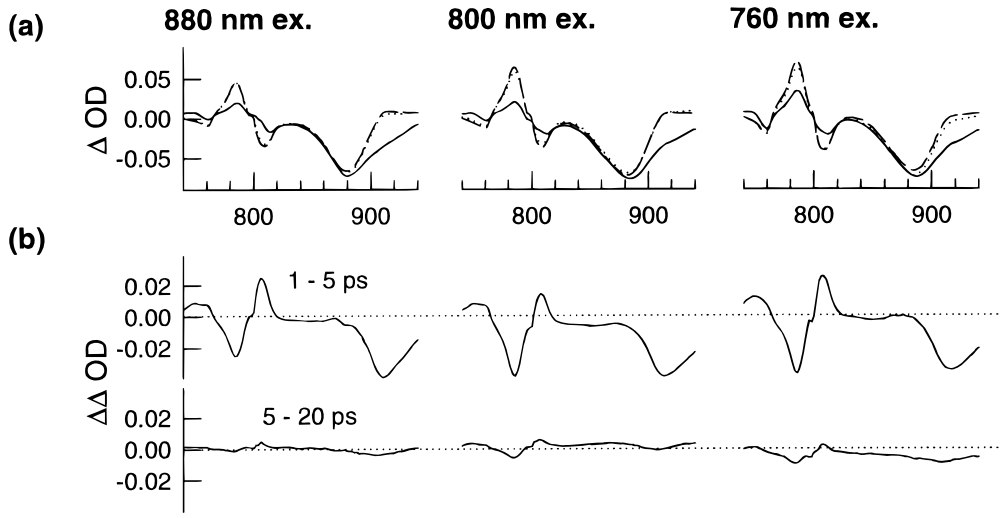

Figure 5. (a) Time-resolved R-26 reaction center absorbance change spectra at 1 (solid line), 5 ± 1 (dotted line), and 20 ± 1 ps (dashed line) after excitation using either 760, 800, or 880 nm pulses. (b) Difference absorbance change spectra with excitation at either 760, 800, or 880 nm. Each spectrum in (b) is the difference between two of the absorbance change spectra at different times shown in (a).

using different excitation conditions at 1, 5, 20, and 40 ps. These spectra are taken from the same data set as the 35 ps spectra of Figure 4 and, thus, are at low time resolution (0.5 ps per point). The 1 ps spectrum in each case is the difference spectrum in the data set with the greatest absorbance decrease due to stimulated emission. As the time constant for the formation of P^+ from P* is about 1.2 ps at low temperatures, one would expect that the changes occurring between 1 and 5 ps would largely be due to formation of $P^+H_A^-$ from P*. Indeed, Figure 5 shows that most of the spectral changes associated with formation of $P^+H_A^-$ take place between 1 and 5 ps. The difference−difference spectra between 1 and 5 ps show a major bleaching decrease around 920 nm due to the decay of the stimulated emission of P* and a derivative shape absorbance change due to the electrochromic shift of the B band resulting from P^+ formation. Note, however, that the difference−difference spectra are not identical. In particular, the ratio of the bleaching near 880 nm to the derivative-like signal centered at 800 nm becomes smaller as higher energy excitation wavelengths are used.

Small-amplitude changes also take place after the first 5 ps using 880 nm excitation, and significantly larger changes are observed after the first 5 ps using 760 and 800 nm excitation. There is further development of the 785, 810, and 880 nm bands normally associated with charge separation between 5 and 20 ps, and the amplitude and shape of these spectral changes are again excitation wavelength dependent. In the case of 880 nm excitation, the difference−difference spectra between 5 and 20 ps have a shape similar to the difference−difference spectra between 1 and 5 ps, but less than 10% of the amplitude. With 760 nm excitation, the 5−20 ps spectra are roughly one-fourth the size of those between 1 and 5 ps.

Discussion

Ultrafast Energy Transfer. There is now a fairly large body of experimental data on energy transfer between H, B, and P in bacterial reaction centers.[16,17,20−23,25,27−30] It appears from this work that H to B energy transfer occurs on the 100 fs or less time scale and that energy transfer from B to P takes place in roughly 100−150 fs, depending somewhat on the species. Similar conclusions can be drawn from the data of Figure 3 in which there are time delays in the formation of P* that are on the order of 100 fs when B is excited and 200 fs when H is excited relative to direct excitation of P. This report and previous reports have also shown that the temperature dependence of the energy transfer rate is weak, changing little between room temperature and 12 K. Once P* is formed, charge separation takes place with a time constant of roughly 1.3 ps at 12 K, forming $P^+H_A^-$.

Are There Alternate Electron-Transfer Pathways? If the above scenario represented a complete picture of energy transfer and charge separation in bacterial reaction centers, then one would expect that on a very rapid time scale (a picosecond or less) the spectral evolution of the system would become independent of the excitation wavelength. Several reports have been published recently that clearly show that the system remains excitation wavelength dependent for many picoseconds.[14,15,24,28] This can be seen also in Figure 4 which shows the spectra of the charge-separated state that result after 35 ps at 12 K. Numerous differences are apparent across the spectrum. Even at room temperature, though the long time spectra at different excitation wavelengths become much more similar, they are not identical, particularly in the regions of H and B absorbance.[24]

One of the possible explanations for the spectral differences observed using different excitation wavelengths is that the reaction center undergoes (at least in part) different photochemistry on the picosecond time scale upon excitation at different wavelengths. This was suggested by the authors of the present report based on room-temperature measurements[24] as well as by Vos et al.[28] in an analysis of high time resolution, low-temperature measurements of excitation wavelength dependence and by Van Brederode et al.[29,30] after analyzing the excitation spectrum and time-resolved absorbance changes in a mutant with a long P* lifetime.

There are a couple of observations that appear to support this interpretation. First, there appears to be less P* formed, when wild-type reaction centers are excited at either 800 or 760 nm than is seen upon direct excitation of P at 880 nm (860 nm at room temperature). The evidence for this can be seen in either Figure 2 or ref 28 at low temperature or in ref 24 at room temperature. In Figure 2, one sees that the red side bleaching at early time appears smaller by 15−20% in the 920 nm region when 800 or 760 nm excitation is used (compared to when 880 nm excitation is used), after normalizing all spectra based on

the final bleaching of the P band due to P^+ formation. This suggests that P^+ is formed by pathways that do not result in P* formation.

This is an important point and worth some scrutiny. One could argue, particularly at low temperature, that a simple comparison of the maximal stimulated emission levels using different excitation wavelengths is not valid. The kinetics of energy transfer and electron transfer are similar enough that some kinetic mixing occurs. In other words, upon 760 or 800 nm excitation, some of the reaction centers have already undergone charge separation on the time scale of P* formation. However, similar results have been obtained in a mutant in which charge separation is much slower.[29] In this mutant, YW(M210), there should be no kinetic mixing. In addition, the YW(M210) mutant also has an excitation spectrum which shows that there is only about half as much integrated fluorescence from P* when H or B is excited than there is when P is excited directly, yet the action spectrum of P^+ formation appears to be the same as the absorbance spectrum, implying that the yield of photochemistry is the same regardless of where excitation occurs. Questions still remain as to why the excitation wavelength effects appear to be much larger in the YW(M210) mutant than in R-26. One possibility is that in the wild type much of the total fluorescence and stimulated emission seen is due to emission from P* formed by charge recombination rather than by energy transfer. This emission would be present as a background regardless of which pathway was used in the formation of the charge-separated state. Thus, while there remain questions about the analysis of stimulated emission in R-26 reaction centers (and presumably wild type), the apparent decrease in the amount of stimulated emission using 760 and 800 nm excitation is probably not an artifact.

If there is less stimulated emission when 760 and 800 nm excitation are used, and if this represents a decrease in P* yield relative to the final P^+ yield (something that does not necessarily follow simply from a decrease in stimulated or spontaneous emission), then some charge separation must be occurring via a pathway that does not involve P*. Obvious candidates for pathway intermediates are charge-separated states formed directly from B* or H*, most probably P^+B^- or B^+H^-.

This brings up the second point in favor of charge separation directly from B* and H*. When one excites in the H or B ground-state absorbance bands, one sees additional absorbance changes in the H and B ground-state bands that could be due to the formation of anion or cation states of these cofactors (Figure 2 and ref 28). Similar observations have also been made in the YW(M210) mutant.[29] It is clear that there are significant differences in the B and H ground-state absorbance regions on the picosecond time scale when charge separation is initiated by excitation at these wavelengths, compared to excitation directly into P.

As appealing as the hypothesis that charge separation occurs directly from B* and H* may be, there are a number of observations that are not easily explained in this way. First, one must appreciate the fact that energy transfer occurs on the 100 fs time scale. If direct charge separation is going to compete with this, it must be extraordinarily rapid—at least as fast as the primary charge separation reaction from P*. Another concern comes from a series of recent measurements performed by this lab on a mutant that lacks the P transition (apparently missing one or both of P's bacteriochlorophylls). Without the background of normal charge separation, it would be easy to pick up small amounts of B^+H^-, yet none was detected.[36] While one can certainly argue that in the mutant the photochemistry is different than in R-26, the fact is that in a mutant where the B and H transitions looked very similar to wild type and the P transition was missing, excitation energy stayed in the reaction center (mostly on B*) for hundreds of picoseconds with no clearly detectable photochemistry occurring. Finally, if one analyzes the R-26 spectra either at room temperature[24] or at low temperature (Figures 2 and 5) looking for changes on the picosecond or longer time scale which represent the decay of B^+H^- or P^+B^- to $P^+H_A^-$, one does not see any kinetic component that shows correlated spectral changes in the H, B, and P bands which correspond in a simple way to $B^+H^- \rightarrow P^+H_A^-$ (recovery of B ground-state absorbance and a decrease in the P ground-state absorbance) or $P^+B^- \rightarrow P^+H_A^-$ (recovery of B, bleaching in H bands). Of course, it could be that these reactions occur very rapidly (a picosecond or faster), and therefore, the intermediates are not easily observed. However, if this was true, one would again expect that the spectral evolution would become excitation wavelength independent on a similarly short time scale.

In addition to the reservations described above, there is another observation that complicates the spectral interpretation and identification of possible intermediate states as B^+H^- and/or P^+B^-. First, at low temperature in R-26 reaction centers, the spectra obtained from exciting in the H, B, and P ground-state transitions remain different even after tens or hundreds of picoseconds. In particular, the spectral changes in the 800 and 760 nm regions remain quite substantial after 35 ps (Figure 4), and at least the excess bleaching at 760 nm when excitation occurs is present for several hundred picoseconds after excitation. (The smaller excitation wavelength dependent bleaching near 800 nm may well persist after several hundred picoseconds as well but is harder to measure on long time scales due to baseline drift.) This suggests that, if one explains the wavelength dependent spectra exclusively in terms of direct charge separation from B* and H*, the intermediate charge-separated or excited states in this process must be stable for a similarly long time. However, $P^+B_A^-$ is thought to have a subpicosecond lifetime at low temperature.[6] There is no reason to believe that $P^+B_B^-$ would be any more stable. Little is known about the state B^+H^- and its lifetime, though it is certainly above P^+H^- in free energy, given the fact that P can be easily oxidized in situ by ferricyanide while B cannot. One might expect that electron transfer from P to B^+ would occur in picoseconds or less, given the rate of other electron-transfer reactions between P, B, and H. Also, when the YW(M210) mutant investigated by Van Brederode et al.[29] is modeled in terms of direct formation of B^+H^-, they find that in order to explain their data P to B^+ electron transfer must occur in less than 10 ps. Thus, it seems unlikely that formation of either of these charge-separated states could explain excitation wavelength dependence observed after several tens to hundreds of picoseconds.

While none of the arguments outlined above strongly argue against the possibility that charge separation directly from B* or H* is in part responsible for the excitation wavelength dependent spectral changes in R-26 reaction centers, they do imply that at least part of the excitation wavelength dependence comes from other sources. On fast time scales (a few picoseconds) it is possible that part of the excitation wavelength dependence comes from vibrational relaxation as suggested at room temperature.[24] On longer time scales (tens to hundreds of picoseconds) vibrational relaxation should be complete, and it probably makes more sense to explain the excitation wavelength dependence in terms of long-lived reaction center heterogeneity. One could argue that the population of reaction

centers which is excited at 800 or 760 nm is spectrally different from those predominantly excited at 880 nm. If this is true, then the long-lived spectra of Figure 4 indicate that excitation at 760 or 800 nm results in selection of a subpopulation of reaction centers that has greater ground-state absorbance at these wavelengths and a somewhat smaller apparent emission (or stimulated emission) level near 920 nm than that seen using 880 nm excitation. Following this logic further, one reason the excitation wavelength dependence is so much more pronounced at low temperature than at room temperature is presumably because at room temperature some of the conformational substates that are static at low temperature are able to interconvert on fast time scales. One result of this line of reasoning is that the heterogeneity in reaction center spectra is not only in the transition energies but also in the oscillator strength of particular bands. Perhaps this is not surprising, given the intensity borrowing thought to occur between chromophore Q_y transitions and the involvement of charge-transfer states in the lowest excited singlet state of P.[37,38] Variations in the interactions between transitions could change the relative oscillator strengths of the B, H, and P bands. Changes in the relative energy of charge-transfer states could alter the amount of emission seen from the excited state of P by changing the displacement of the excited state relative to the ground state.

In summary, the long-lived excitation wavelength dependence may be most easily explained in terms of substantial spectral heterogeneity in the reaction center population rather than alternate charge separation pathways. Once one accepts this premise, much of the unexpected excitation wavelength dependence, past the first picosecond where energy transfer occurs, can be explained in terms of spectrally selective excitation of reaction centers.

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References and Notes

(1) Kirmaier, C.; Holten, D. *Photosynth. Res.* **1987**, *13*, 225.

(2) Feher, G.; Allen, J. P.; Okamura, M. Y.; Rees, D. C. *Nature* **1989**, *339*, 111.

(3) Parson, W. W. In *Chlorophylls;* Scheer, H., Ed.; CRC Press: Boca Raton, FL, 1991; p 1153.

(4) Martin, J.-L.; Vos, M. H. *Annu. Rev. Biophys. Biomol. Struct.* **1992**, *21*, 199.

(5) Kirmaier, C.; Holten, D. In *The Photosynthetic Reaction Center;* Deisenhofer, J., Norris, J. R., Eds.; Academic Press: San Diego, 1993; Vol. II, p 49.

(6) Zinth, W.; Kaiser, W. In *The Photosynthetic Reaction Center;* Deisenhofer, J., Norris, J. R., Eds.; Academic Press: San Diego, 1993; Vol. II, p 71.

(7) Woodbury, N. W.; Allen, J. P. In *Anoxygenic Photosynthetic Bacteria;* Blankenship, R. E., Madigan, M. T., Bauer, C. E., Eds.; Kluwer Academic Publishers: Dordrecht, 1995; Vol. 2, p 527.

(8) Woodbury, N. W.; Peloquin, J. M.; Alden, R. G.; Lin, X.; Lin, S.; Taguchi, A. K. W.; Williams, J. C.; Allen, J. P. *Biochemistry* **1994**, *33*, 8101.

(9) Allen, J. P.; Williams, J. C. *J. Bioenerg. Biomembr.* **1995**, *27*, 275.

(10) Vos, M. H.; Jones, M. R.; Hunter, C. N.; Breton, J.; Martin, J.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12701.

(11) Vos, M. H.; Rappaport, F.; Lambry, J.-C.; Breton, J.; Martin, J.-L. *Nature* **1993**, *363*, 320.

(12) Vos, M. H.; Jones, M. R.; Hunter, C. N.; Breton, J.; Lambry, J.-C.; Martin, J.-L. *Biochemistry* **1994**, *33*, 6750.

(13) Stanley, R. J.; Boxer, S. G. *J. Phys. Chem.* **1995**, *99*, 859.

(14) Peloquin, J. M.; Lin, S.; Taguchi, A. K. W.; Woodbury, N. W. *J. Phys. Chem.* **1996**, *100*, 14228.

(15) Peloquin, J. M.; Lin, S.; Taguchi, A. K. W.; Woodbury, N. W. *J. Phys. Chem.* **1995**, *99*, 1349.

(16) Breton, J.; Martin, J.-L.; Migus, A.; Antonetti, A.; Orszag, A. In *Ultrafast Phenomena V;* Fleming, G. R., Siegman, A. E., Eds.; Springer-Verlag: 1986; p 393.

(17) Breton, J.; Martin, J.-L.; Migus, A.; Antonetti, A.; Orszag, A. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 5121.

(18) Johnson, S. G.; Tang, D.; Jankowiak, R.; Hayes, J. M.; Small, G. J. *J. Phys. Chem.* **1990**, *94*, 5849.

(19) Vos, M. H.; Lambry, J.-C.; Robles, S. J.; Youvan, D. C.; Breton, J.; Martin, J.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 613.

(20) Jia, Y.; Jonas, D. M.; Joo, T.; Nagasawa, Y.; Lang, M. J.; Fleming, G. R. *J. Phys. Chem.* **1995**, *99*, 6263.

(21) Stanley, R. J.; King, B.; Boxer, S. G. *J. Phys. Chem.* **1996**, *100*, 12052.

(22) Haran, G.; Wynne, K.; Moser, C. C.; Dutton, P. L.; Hochstrasser, R. M. *J. Phys. Chem.* **1996**, *100*, 5562.

(23) Jonas, D. M.; Lang, M. J.; Nagasawa, Y.; Joo, T.; Fleming, G. R. *J. Phys. Chem.* **1996**, *100*, 12660.

(24) Lin, S.; Taguchi, A. K. W.; Woodbury, N. W. *J. Phys. Chem.* **1996**, *100*, 17067.

(25) Breton, J.; Martin, J.-L.; Fleming, G. R.; Lambry, J.-C. *Biochemistry* **1988**, *27*, 8276.

(26) Jean, J. M.; Chan, C.-K.; Fleming, G. R. *Isr. J. Chem.* **1988**, *28*, 169.

(27) Wynne, K.; Haran, G.; Reid, G. D.; Moser, C. C.; Dutton, P. L.; Hochstrasser, R. M. *J. Phys. Chem.* **1996**, *100*, 5140.

(28) Vos, M. H.; Breton, J.; Martin, J.-L. *J. Phys. Chem. B* **1997**, *101*, 9820.

(29) Van Brederode, M. E.; Jones, M. R.; Van Mourik, F.; Van Stokkum, I. H. M.; Van Grondelle, R. *Biochemistry* **1997**, *36*, 6855.

(30) Van Brederode, M. E.; Jones, M. R.; Van Grondelle, R. *Chem. Phys. Lett.* **1997**, *268*, 143.

(31) Feher, G.; Okamura, M. Y. In *The Photosynthetic Bacteria;* Clayton, R. K., Sistrom, W. R., Eds.; Plenum Press: New York, 1978; p 349.

(32) Woodbury, N. W.; Lin, S.; Lin, X.; Peloquin, J. M.; Taguchi, A. K. W.; Williams, J. C.; Allen, J. P. *Chem. Phys.* **1995**, *197*, 405.

(33) Okamura, M. Y.; Isaacson, R. A.; Feher, G. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3491.

(34) Holten, D.; Windsor, M. W.; Parson, W. W.; Thornber, J. P. *Biochim. Biophys. Acta* **1978**, *501*, 112.

(35) Maroti, P.; Kirmaier, C.; Wraight, C.; Holten, D.; Pearlstein, R. M. *Biochim. Biophys. Acta* **1985**, *810*, 132.

(36) Jackson, J. A.; Lin, S.; Taguchi, A. K. W.; Williams, J. C.; Allen, J. P.; Woodbury, N. W. *J. Phys. Chem. B* **1997**, *101*, 5747.

(37) Parson, W. W.; Warshel, A. *J. Am. Chem. Soc.* **1987**, *109*, 6152.

(38) Warshel, A.; Parson, W. W. *J. Am. Chem. Soc.* **1987**, *109*, 6143.