Energy Transfer in *Rhodobacter sphaeroides* Reaction Centers with the Initial Electron Donor Oxidized or Missing

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Energy transfer between chromophores in the reaction center of *Rhodobacter sphaeroides* R-26 with and without the initial donor oxidized and in reaction centers from the strain VR(L157) that has the mutation Val to Arg at L157 has been investigated using femtosecond transient absorption spectroscopy. Pigment extractions and an analysis of the ground-state absorbance spectrum indicate that VR(L157) reaction centers have a substantial reduction in the amount of bacteriochlorophyll present per reaction center, due to the loss of one or both of the bacteriochlorophylls in the initial electron donor (P). The Q_Y transition bands of the bacteriochlorophyll monomers (B) and the bacteriopheophytins (H) in reaction centers from the three reaction center samples (R-26 with and without P oxidized and VR(L157)) were selectively excited using 150 fs duration, 5 nm bandwidth pulses, and transient absorbance spectra were recorded. Quenching of the lowest excited state of the bacteriochlorophyll monomer (B*) occurs in hundreds of femtoseconds in R-26 reaction centers either with or without P initially oxidized. Selective excitation of B in VR(L157) reaction centers results in a nanosecond lifetime B* excited state. Neither charge separation nor fast quenching of B* is observed in VR(L157) reaction centers, contrary to what one might expect from predictions of the energetics of B* relative to charge-separated states such as B^+H^-. The fluorescence spectrum of B* in this mutant is similar in width to the absorbance spectrum of B and is centered near 801 nm (the mutant's ground-state B band peaks at 796 nm). Picosecond fluorescence measurements of VR(L157) reaction centers show that fluorescence decay from B* is multiexponential and occurs on the order of nanoseconds. From these results, it is concluded that P is required for fast quenching of B but that the oxidation state of P does not strongly affect the quenching process. This suggests that spectral or orbital overlap between B and P does not limit the ultrafast rate of energy transfer between these two cofactors. One possibility is that nuclear motion limits the rate of energy transfer.

Introduction

Light absorbed by reaction centers of purple nonsulfur bacteria results in formation of the lowest excited singlet state (P*) of a bacteriochlorophyll dimer (P) either directly or through rapid energy transfer between cofactors. An electron is then transferred from P* to a bacteriopheophytin monomer (H_A) in a few picoseconds via a monomer bacteriochlorophyll (B_A) (for reviews, see refs 1−6). The reaction forming the radical pair state $P^+H_A^-$ has a yield of near unity and is one of the fastest intermolecular reactions known.[7] The structure of the bacterial reaction center has been determined in two species, *Rhodobacter (Rb.) sphaeroides* and *Rhodopseudomonas viridis*.[8−12] The structure contains two symmetrically related groups of cofactors referred to by the subscripts A and B. Subscript A denotes the cofactors on the branch active in charge separation. Very little charge separation is thought to occur along the B branch.[4,6,13]

Experiments using femtosecond transient spectroscopy at both room temperature and 10 K have shown that excitation initially absorbed by the monomer bacteriochlorophylls is transferred to P on the 100 fs time scale.[14−20] Energy transfer from H* to B is also extremely rapid.[20,21]

Energy transfer between weakly coupled pigments is often described in terms of dipole−dipole interactions between the lowest energy transitions of the two molecules using the Förster model.[22] This model assumes localization of electronic transitions on individual pigments, as well as rapid vibrational relaxation and dephasing on the time scale of energy transfer. Femtosecond transient absorption studies have shown that P* does not undergo complete vibrational relaxation and dephasing for at least a picosecond after excitation.[23,24] In addition, calculations using the Förster model resulted in rate constants for energy transfer between cofactors that were at least an order of magnitude slower than those obtained by experiment,[25] suggesting that energy transfer in the reaction center may occur by a different mechanism, though there is still disagreement on this issue.[19,26,27]

Other questions remain regarding the photochemistry of reaction centers that have been excited directly in the monomer bacteriochlorophyll or bacteriopheophytin transitions. One might expect that the energetics of charge separation starting from one of the excited bacteriochlorophyll monomers or bacteriopheophytins and forming B^+H^- would be about as favorable as the initial electron transfer from the excited special pair forming $P^+H_A^-$, judging from excited-state energies and in vitro oxidation potentials of bacteriopheophytin and bacteriochlorophyll. An analysis of the amount of P* created during charge separation when B or H is specifically excited suggests that roughly 20% of charge separation does not occur via the excited singlet state of P at room temperature.[20] This could be due to charge separation directly from H* or B* via states such as B^+H^- or P^+B^-. Consistent with this idea, direct excitation into H gives additional bleaching in the H and the B bands

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shortly after excitation (though this may be due to incomplete vibrational relaxation).[20]

Another interesting aspect of the early photochemistry in this system is that the rate of energy transfer from B* to P is nearly independent of whether P is oxidized or at moderate potential.[17] Why P and P^+ would be nearly equally good quenchers remains a mystery.

In this work, we consider energy transfer and excitation quenching in R-26 reaction centers, both with and without P oxidized, and in reaction centers from VR(L157), a strain in which the Q_Y absorbance transition of the special pair appears to be missing, apparently due to loss of some or all of the special pair bacteriochlorophylls.

Materials and Methods

Reaction Center Mutagenesis, Expression, and Isolation. Reaction centers from *Rb. sphaeroides* carotenoidless strain R-26 were isolated as described elsewhere[28] and suspended in 50 mM Tris-HCl (pH 8.0), 0.025% LDAO, 1 mM EDTA, 0.5 mM terbutryne, 10 μM PMS, and 100 μM ascorbate. For samples in which P was initially oxidized to P^+, 75−180 mM $K_3Fe(CN)_6$ was added to the solution as noted in the figure legends.

Construction of the VR(L157) mutant, in which the codon for Val L157 (GTG) was changed to Arg (CGG), was accomplished by the megaprimer method.[29] The pRKSCH expression vectors[30] and ΔLM1.1 deletion strain,[31] which is derived from the wild-type 2.4.1 strain, have been described previously. Cells were grown semiaerobically in the dark. Reaction centers were isolated and suspended in 15 mM Tris-HCl (pH 8), 0.025% LDAO, and 1 mM EDTA as described in refs 31 and 30.

Pigment Extractions. Pigment extractions were performed on isolated reaction centers of the strain VR(L157) as described previously.[32] The ratio of bacteriochlorophyll:bacteriopheophytin was determined using the extinction coefficients from Straley et al.[32] and from van der Rest and Gingras.[33] An error of approximately 0.05 in the bacteriochlorophyll:bacteriopheophytin ratio was determined by analysis of multiple wild-type preparations. The ratio reported for VR(L157) reaction centers represents an average of several measurements made on the preparation used for the other spectroscopic measurements.

Transient Absorption Spectroscopy. A pump−probe transient absorbance spectrophotometer was used to obtain time-resolved absorbance difference spectra as described previously.[34,35] Spectrally narrow excitation pulses (4−5 nm full width at half-maximum) were polarized at the magic angle with respect to the probe pulse. The excitation pulses had an energy of 2−4 μJ pulse over a roughly 2 mm area, causing the excitation of 15−20% of the reaction centers in the sample. The pulse duration was 150 fs and the repetition rate was 540 Hz. The wavelength-dependent dispersion in the delay between the pump and probe pulses was corrected empirically as described previously.[24,35] Samples were loaded into a spinning wheel with an optical path length of 2.5 mm, giving a final optical density between 0.7 and 1.0 at 800 nm.[36] All measurements were performed at room temperature.

Time-Correlated Single-Photon Counting Measurements. Fluorescence measurements were carried out on VR(L157) reaction centers suspended in 15 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.025% LDAO. The optical density at 800 nm was 0.18 in a 2 mm path length cell. This is somewhat more dense than optimum, but lower concentrations resulted in poor quality spectra. Because of the optical density, there may be some self-absorption near 796 nm (about 30%), resulting in fluorescence spectra that are slightly red-shifted. The time-correlated single-photon counting apparatus has been described previously.[36] For this work, approximately 10-ps excitation pulses at 3.8 MHz from a cavity-dumped, synchronously pumped, styryl-9 dye laser at 789 nm (Spectra Physics) were used. The dye laser was pumped by an Antares mode-locked Nd:Yag laser (Coherent). Approximately 20−30 mW/cm^2 excitation was used. The monochromator, emission detector, and timing circuitry have been described previously.[36,37] The full width at half-maximum of the instrument response function was 80−100 ps.

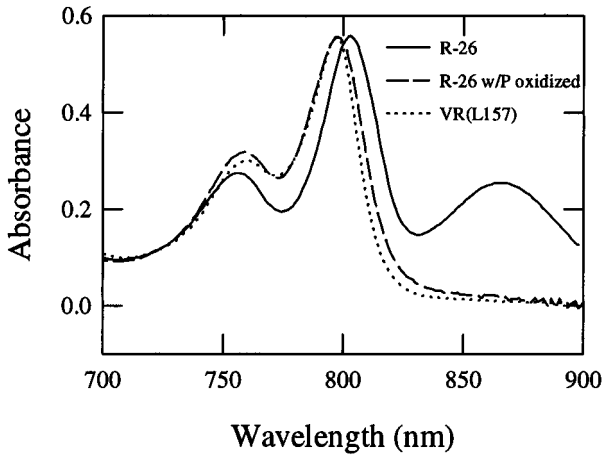

Figure 1. Room temperature, ground-state absorbance spectra of isolated reaction centers from *Rb. sphaeroides* strain R-26 with (dashed line) and without (solid line) P initially oxidized by the addition of 75 mM ferricyanide. The ground-state spectrum of the reaction center mutant VR(L157) (dotted line) is also shown.

Fluorescence decays taken using the 789 nm excitation pulses described above were analyzed by fitting to a sum of exponential decay terms convoluted with the measured instrument response function. This was done simultaneously using data taken at emission wavelengths 800, 805, and 810 nm.[36] The reduced global χ^2 error for the fit presented was 1.17.

The steady-state fluorescence spectrum of VR(L157) reaction centers was obtained from integrated emission measurements using the same time-correlated single photon counting apparatus described above and excitation at 786 nm. Emission data were taken at 1 nm intervals between 795 and 840 nm with a spectral resolution of 1 nm.

Results

Ground-State Absorbance Spectra. Figure 1 shows the ground-state absorbance spectra taken at room temperature for the three types of reaction center samples used in this study. R-26 reaction centers with and without P oxidized are shown without normalization. VR(L157) reaction centers were normalized to the P-oxidized R-26 reaction centers at 797 nm.

R-26 reaction centers without P initially oxidized show major transitions at 760, 802, and 865 nm that correspond predominantly to Q_Y transitions for H, B, and P, respectively. Oxidation of P in R-26 reaction centers is known to result in loss of the 865 nm band of the initial electron donor as well as a blue shift of the 800 nm transition.[1] For this study, it was important to oxidize P as completely as possible, to investigate energy transfer from B* to P^+ with as little background energy transfer to unoxidized P as possible. Because of this, many of the spectroscopic experiments were performed using rather high ferricyanide concentrations. The data of Figure 1 were taken with a ferricyanide concentration of 75 mM. There was essentially no further bleaching of the Q_Y band of P at ferricyanide concentrations up to 180 mM.

The spectrum of the reaction centers from the mutant, VR-(L157), has less than 5% of the oscillator strength at 865 nm that is present in the P band of either wild-type or R-26. In fact, this spectrum is strikingly similar to the R-26 reaction center spectrum when P is oxidized. Interestingly, the similarity extends to the blue-shift in the position of the monomer bacteriochlorophyll peak, which occurs either in oxidized R-26 reaction centers or in the VR(L157) mutant. Note that the almost exact alignment between VR(L157) and oxidized R-26 in Figure 1 is probably fortuitous, since the monomer bacteriochlorophyll band position in R-26 reaction centers with P oxidized is not the same as that in wild-type reaction centers (from which VR(L157) was derived). VR(L157) reaction centers also show a decreased spectral width of the 800 nm band relative to either oxidized or moderate potential R-26 reaction centers (Figure 1) or relative to wild-type reaction centers with P either oxidized or not oxidized (data not shown).

To test whether the VR(L157) mutant actually lacks one or both of the bacteriochlorophylls which make up P, pigment extractions were performed (described in Methods), resulting in a bacteriochlorophyll:bacteriopheophytin ratio of 1.25 ± 0.05. (Wild-type values of the ratio obtained using this method are 1.90 ± 0.05.) This is consistent with a substantial loss of bacteriochlorophyll from the reaction center. However, this value is significantly greater than the ratio of 1.00 expected for complete loss of the two bacteriochlorophylls in the initial electron donor. Thus, complete loss of both of the special pair bacteriochlorophylls may not have occurred in all reaction centers.

Absorbance Change Kinetics Using 800 and 760 nm Excitation. The 800 nm transient absorbance changes observed using excitation at 800 nm are shown in the bottom panel of Figure 2. Each kinetic trace results from 100 time points at 55 fs intervals. The decay curves were normalized at the time of maximum bleaching. Pumping and probing at 800 nm, the time dependence of the absorbance changes for R-26 reaction centers with and without P initially oxidized are very similar during the first picosecond. Both reach a maximum absorbance decrease in hundreds of femtoseconds, and after an additional several hundred femtoseconds the absorbance decrease at 800 nm recovers in both samples. Charge separation results in an absorbance decrease at 800 nm with a time constant of several picoseconds in the case of R-26 reaction centers with P at moderate potential. This longer time scale absorbance change is absent in the sample with P oxidized, because $P^+H_A^-$ formation is not possible. In the VR(L157), the initial absorbance decrease at 800 nm recovers only slightly on the several picosecond time scale. Longer time scale measurements have shown that the initial bleaching near 800 nm recovers with an overall lifetime of roughly 800 ps (Figure 3).

The kinetic traces at 760 nm using 760 nm excitation for R-26 and VR(L157) are shown in the top panel of Figure 2. The time course of the 760 nm absorbance changes for R-26 reaction centers at moderate ambient redox potential displays a fast absorbance decrease upon light absorption followed by a recovery in several hundred femtoseconds. This recovery is followed by a slower absorbance decrease on the several picosecond time scale. The kinetics for VR(L157) show a fast initial absorbance decrease upon excitation followed by a partial recovery in several hundred femtoseconds to a level that is constant on the picosecond time scale. Measurements using 760 nm excitation were not performed for R-26 reaction centers with P initially oxidized.

Spectral Evolution of Energy and Electron Transfer. Figure 3 shows the absorbance change spectra using 800 nm excitation at selected times for each reaction center sample investigated. For the R-26 reaction centers with and without P initially oxidized, the spectra were taken on a 10 ps time scale. For the VR(L157) reaction centers, the spectra were taken on the 1 ns time scale.

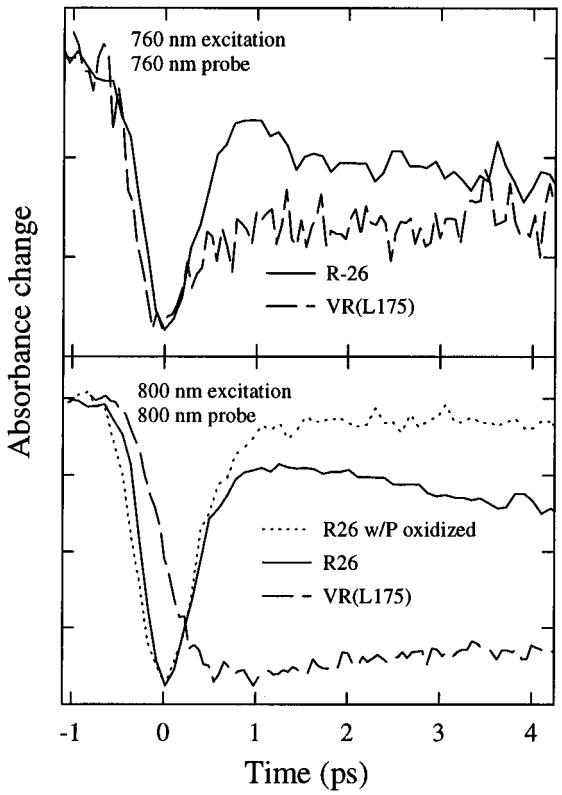

Figure 2. Absorbance change kinetics are shown of R-26 reaction centers with (solid line) and without (dotted line) P initially oxidized by 180 mM ferricyanide and of VR(L157) (dashed line) reaction centers. Upper panel: both the excitation and probe pulses are at 760 nm. Lower panel: both the excitation and probe pulses are at 800 nm. Curves are normalized at the maximum bleaching in each panel. Data points were recorded at 55 fs intervals.

The 0 ps spectrum of R-26 reaction centers without P initially oxidized shows an absorbance decrease centered at 802 nm with a spectral full width at half-maximum (fwhm) of about 15 nm, a broad weak absorbance decrease centered at about 870 nm, and a broad absorbance increase below 780 nm. The bleaching at 870 nm in this sample reaches a maximum in several hundred femtoseconds, and by 7 ps, the center has shifted to 865 nm. At 7 ps most of the apparent bleaching between 900 and 950 nm due to stimulated emission has disappeared. Between 1 and 7 ps, the absorbance near 810 nm decreases, and an absorbance increase occurs centered at about 780 nm. Similar results have been observed previously for R-26 reaction centers using 800 nm excitation.[20]

The 0 ps spectrum for R-26 reaction centers with P initially oxidized shows a 17 nm fwhm absorbance decrease centered at 798 nm, a small broad absorbance decrease centered at about 870 nm (presumably due to unoxidized reaction centers, see below), and a small broad absorbance increase below 780 nm. The bleaching centered at 798 nm has largely recovered by 1 ps, though there also appears to be some ground-state recovery on a longer time scale as well. After several picoseconds, the spectrum is essentially identical with that of reaction centers in which P has not been chemically oxidized (though the magnitude of the signal is much smaller relative to the total reaction center sample absorbance), indicating that a small fraction (10−15%) of R-26 reaction centers were not in the state P^+ during the

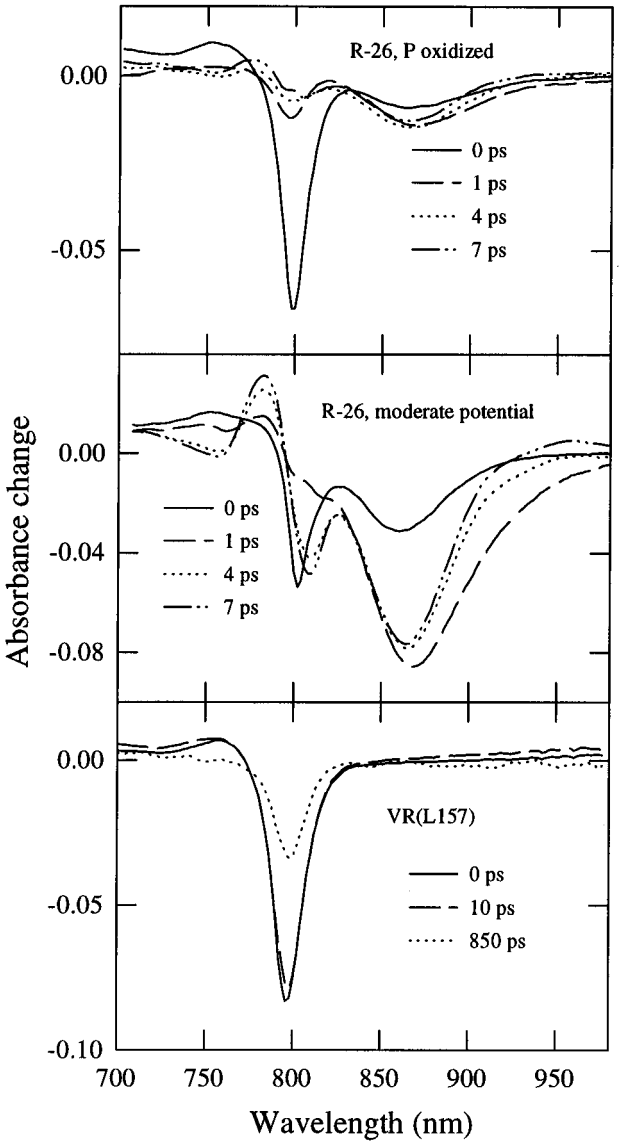

Figure 3. Time-resolved absorbance spectra are shown of R-26 reaction centers with (upper panel) and without (middle panel) P initially oxidized by 180 mM ferricyanide and of VR(L157) reaction centers (lower panel) using excitation at 800 nm. Time zero is defined as the point when the maximal absorbance decrease at the excitation wavelength is achieved. Data points were recorded at 2 nm intervals.

measurement. These measurements were repeated at several ferricyanide concentrations between 75 and 180 mM with essentially identical results. The results using 180 mM ferricyanide are shown in the figures, since this concentration was expected to yield the lowest amount of unoxidized P. There is no evidence for 10−15% unoxidized P in the ground state spectrum of R-26 reaction centers at 75 mM ferricyanide (Figure 1) or at higher ferricyanide concentrations. Remeasurement of the absorbance spectrum of chemically oxidized reaction centers after exposure to 800 nm light showed that a fraction (10−15%) of the reaction centers had recovered the long-wavelength transition associated with P (data not shown). These photoconverted reaction centers could not be completely reoxidized with additional ferricyanide. (Addition of an extra 40 mM ferricyanide at this point only resulted in a roughly 20% decrease in the 860 nm band of P. Increasing the ferricyanide concentration beyond this had little effect). The photoconversion of chemically oxidized reaction centers with 800 nm light represents an interesting phenomenon which has not previously been reported. It is possible that the hydrogen bonding between P

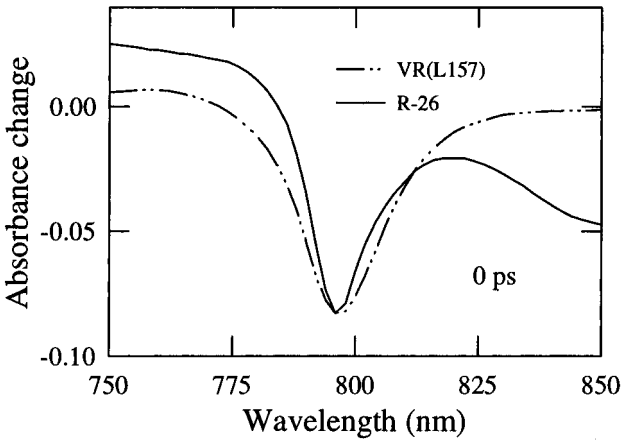

Figure 4. Time-resolved absorbance spectra of VR(L157) reaction centers (dashed line) and of R-26 reaction centers without P initially oxidized (solid line) at 0.0 ps using excitation at 800 nm. Time zero is defined as the point when maximal absorbance decrease at the excitation wavelength is achieved. Data points were recorded at 2 nm intervals.

and the surrounding protein has been altered, such as in various mutants with high oxidation potentials.[38] However, since it apparently only affects a small fraction of the total reaction centers, the analysis of the bulk of the oxidized reaction centers was continued, and a further investigation of the photoconverted reaction centers was left for a later time.

Upon excitation at 800 nm, VR(L157) reaction centers show a 19 nm FWHM bleaching centered at 796 nm (Figure 3, bottom panel). Approximately 60% of this absorbance decrease has recovered by 850 ps. There is also a broad absorbance increase centered at about 760 nm initially. This absorbance increase has essentially disappeared by 850 ps.

The difference in early time bleaching between R-26 and VR-(L157) reaction centers is shown more clearly in Figure 4. In both cases the bleaching is initially centered close to the excitation wavelength, but the width of the bleaching is about 4 nm broader in the case of VR(L157) reaction centers.

Figure 5, lower panel, shows the time-resolved absorbance spectra for R-26 reaction centers without P initially oxidized using 760 nm excitation. The spectra of both samples were taken on a 5 ps time scale. At 0 ps, R-26 reaction centers show ground-state bleaching at both 760 and 800 nm and broad bleaching centered at 870 nm, as was shown previously for the 760 nm excitation.[20] After 4 ps in R-26 reaction centers, the difference spectrum is essentially the same for either 800 or 760 nm excitation.

At 0 ps, 760 nm excitation of VR(L157) reaction centers results in two overlapping absorbance decrease bands centered at 760 and 796 nm (Figure 5, upper panel). By 1 ps, the band at 760 nm has decreased and appears to have shifted to longer wavelengths, causing the band centered at 796 nm to become broader on the short wavelength side. This asymmetric bleaching centered at 796 remains constant on the time scale of 5 ps with the shorter wavelength shoulder decreasing slightly.

Steady State and Time-Resolved Fluorescence from the Strain VR(L157). Figure 6 shows the steady-state fluorescence for VR(L157) reaction centers using 786 nm excitation. Although VR(L157) reaction centers have an absorbance maximum at 796 nm, an excitation wavelength of 786 nm was used to minimize scattered light in the wavelength region of the fluorescence measurements. The fluorescence spectrum measured in this way has a maximum at 801 nm (Figure 6), implying a very small Stokes shift for the monomer bacteriochlorophyll transitions (the fluorescence peaks about 5 nm to the red of the absorbance peak). A similar result has been found

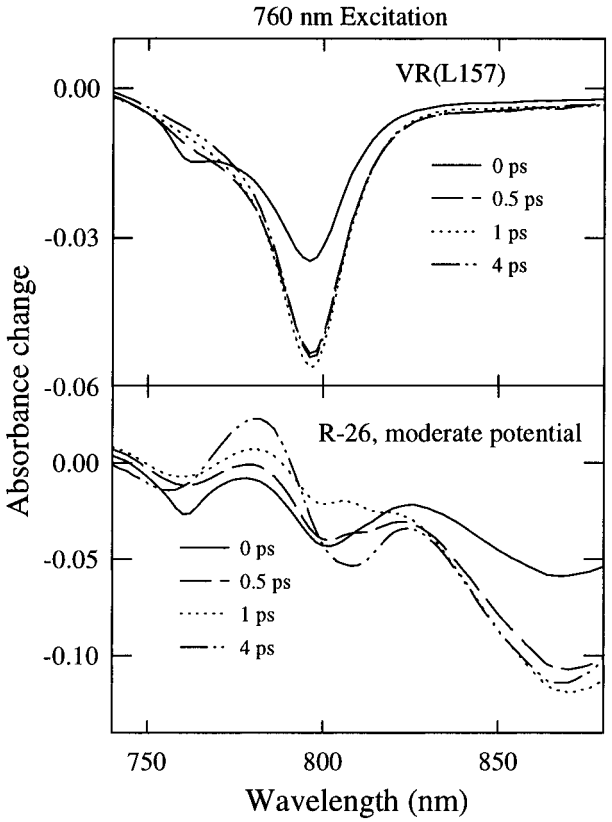

Figure 5. Time-resolved absorbance spectra of reaction centers from VR(L157) (upper panel) and R-26 (lower panel) without P initially oxidized using excitation at 760 nm. Time zero is defined as the point when maximal absorbance decrease at the excitation wavelength is achieved. Data points were recorded at 2 nm intervals.

for another reaction center mutant lacking the P absorbance transition at 865 nm by Boxer and co-workers (personal communication, the mutant is described in ref 39).

Figure 7 shows the fluorescence decay of reaction centers from the VR(L157) mutant at 295 K using 789 nm excitation and 800 nm detection. Also shown are the results of globally fitting fluorescence decays at 800, 805, and 810 nm to a sum of three-exponential decay components.[36] A statistically inferior fit resulted when only one or two exponential decay components were used.

Discussion

Spectroscopy of VR(L157) Reaction Centers. Figure 1 demonstrates that it is possible to generate mutants that completely lack the normal spectral signature of P. VR(L157) is a mutant with at least a significant fraction, though perhaps not all, of the bacteriochlorophyll associated with P missing. Spectroscopically (but not functionally), this mutant is similar to wild-type reaction centers in the state P^+: the main infrared transition of P is absent, and the monomer bacteriochlorophyll transition is blue-shifted (see also ref 39 for a previously reported mutant that lacks the 865 nm band of P). Because B* is long-lived in this mutant, it has been possible to observe both the ground-state bleaching and excited-state absorption spectrum of B (Figure 3) as well as B*'s fluorescence spectrum (Figure 6). The absorbance changes associated with B* are quite similar to those reported for bacteriochlorophyll in solution.[40]

The Role of P in Quenching the Excited State of B. As has been shown previously, excitation specifically into the 800 nm band of the reaction center monomer bacteriochlorophylls results in energy transfer from B to P on the time scale of hundreds of femtoseconds.[15,17−20] The evidence for this comes

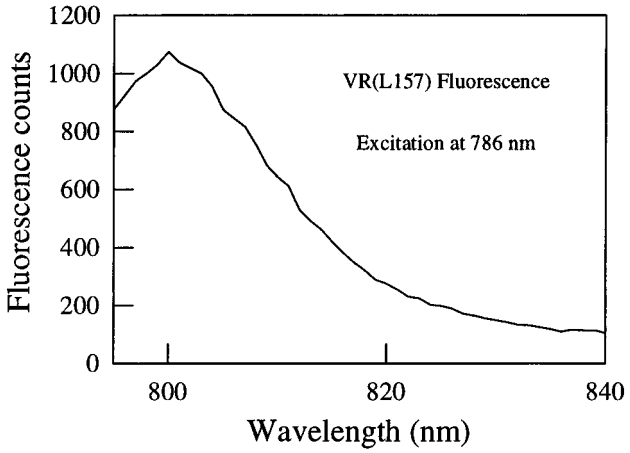

Figure 6. Steady-state fluorescence spectrum of VR(L157) reaction centers. The excitation wavelength was 786 nm. Data points were recorded at 1 nm intervals.

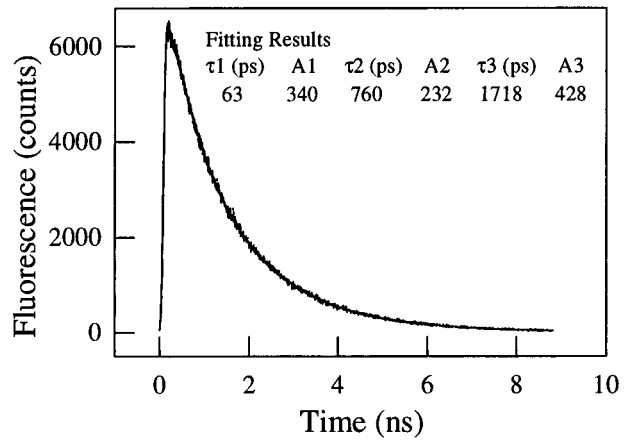

Figure 7. Emission decay kinetics of VR(L157) reaction centers measured by time-correlated single photon counting. Photon counts were accumulated in 2000 channels covering 10 ns. The value shown at each time is the total number of photons detected in the corresponding 5 ps interval. Excitation was 789 nm and detection was 800 nm. Similar results were obtained for fluorescence detected at 805 and 810 nm. Sample conditions and other details of the apparatus are given in the Materials and Methods Section.

from measurements such as those shown in Figures 2 and 3 where one observes a rapid decrease in the initial bleaching of the 800 nm monomer band and a concurrent increase in the bleaching of the 865 nm band of P and the stimulated emission in the 900−950 nm region from P*. Surprisingly, oxidation of P to P^+ has little effect on the rate of disappearance of the 800 nm bleaching (see ref 17 and also Figures 2 and 3). This implies that the excited singlet state of B is being quenched at nearly the same rate when P is in the state P^+ as it is when P is at moderate potential and can form P*.

This observation raises a couple of interesting questions. First, is P itself required for the rapid decay of the monomer bacteriochlorophyll excited singlet state? The answer is also shown in Figures 2 and 3 where one can see that in a mutant that lacks the spectral signature of P, but otherwise appears spectrally similar to wild-type, the bleaching in the 800 nm bands of the monomer bacteriochlorophylls has a lifetime of roughly 800 ps, more than 3 orders of magnitude longer than the lifetime of B* in the presence of either P or P^+. This is further supported by the large relative amplitudes of the 800 ps and 1.7 ns components in the time-resolved fluorescence data from VR(L157) (Figure 7). This leads to a second, more difficult question. Why does chemical oxidation of P result in so little change in the overall rate of energy transfer between B

and P and what does this imply about the mechanism of the ultrafast energy transfer reaction?

Energy Transfer Mediated by Weak Dipole−Dipole Interactions. The most commonly used theory of energy transfer between weakly coupled chromophores is the Förster energy transfer theory. This mechanism utilizes dipole−dipole coupling between nearly resonant transitions of two chromophores in order to transfer energy. The validity of this theory in describing the ultrafast B* to P energy transfer reaction has been discussed previously with mixed conclusions as to whether such a rapid reaction could be accommodated within the weak coupling limit.[19,25−27] The rate of energy transfer in this reaction depends directly on the energy resonance condition between the donor and acceptor which is expressed as an overlap integral between the fluorescence spectrum of the donor and the absorbance spectrum of the acceptor. The theoretical rate also depends on the relative orientation of the transition dipoles of the donor and acceptor molecules.

The absorbance spectrum of P changes dramatically when it is oxidized (Figure 1). The primary near-infrared transition of P essentially disappears. It has been suggested that the upper exciton band of P plays a critical role in the energy transfer mechanism from B* to P.[18,25] This transition is thought to give rise to an absorbance band on the red side of the monomer bacteriochlorophyll band and to overlap most strongly with the fluorescence spectrum of B*. One would expect the upper exciton transition of P to disappear when P is oxidized since it shares the same ground state as the lower exciton band. P^+ may have a broad absorbance throughout this spectral region (this cannot be measured independently due to the effects of P^+ formation on the absorbance spectra of neighboring chromophores). However, it is unlikely (though not impossible) that the spectral overlap integral is very similar in oxidized and moderate potential conditions.

There is also no compelling reason to believe that the orientation of the dominant near-infrared transition dipole moment for the P^+ to P^{+}* transition would be the same as that of the P to P* transition. Thus, both the expected change in spectral overlap and the expected change in the transition dipole moment orientation upon oxidation of P imply that according to the Förster energy transfer theory one should see a much larger difference between the rates of the B* to P and B* to P^+ energy transfer reactions than is observed. This, and the ultrafast speed and temperature dependence of the energy transfer,[19,25] together argue against a Förster mechanism for this reaction.

Energy Transfer Mediated by Electron Exchange Interactions. The other commonly used theoretical description of energy transfer has been formulated by Dexter.[41] In this view, the dominant interaction between the two chromophores is electron exchange. The rate of energy transfer by electron exchange is proportional to the product of the electrostatic interaction between the two molecules and spectral overlap described above. Looked at in another way, the rate of energy transfer should depend on orbital overlap between the LUMO of the donor and the LUMO of the acceptor and between the HOMOs of the donor and acceptor chromophores (for other possible electron exchange mechanisms, see below). As with the Förster mechanism, one would expect that oxidizing the acceptor chromophore would substantially alter the HOMO and LUMO energies, and therefore, the resonance condition required for the exchange. Thus, it is not clear why oxidation of P would not substantially change the rate of energy transfer between B* and P if this rate was limited by the electronic coupling required for electron exchange. Note that energy transfer may well be mediated by electron exchange, however, given the insensitivity of the rate to the electronic state of P, the electronic coupling required for rapid exchange may not be the rate-limiting aspect of the process.

Energy Transfer Limited by Nuclear Motion. It is possible that something other than the electron exchange event itself is rate limiting. We have previously suggested that both energy and electron transfer in the reaction center may occur near the adiabatic limit and that the rate of nuclear motion in the system may limit the overall rate of both processes.[20] In this view, the electronic coupling between the donor and acceptor is large enough (in both P and P^+) so that what limits the reaction rate is the time required to achieve the proper nuclear configuration for an isoenergetic electron exchange to occur. This time should be shorter in the case of energy transfer than in the case of electron transfer since electron transfer is usually accompanied by a larger nuclear displacement than energy transfer.[20] This is what is observed: the rate of energy transfer is in the hundreds of femtoseconds range, and the overall rate of electron transfer is several picoseconds. This would also explain why oxidation of P has so little effect on the energy transfer from the monomer bacteriochlorophylls to the special pair. The resonance condition between B and P could change substantially upon oxidation of P, but as long as it remained strong enough so that nuclear motion was still rate limiting, there would be little effect on the overall rate of energy transfer in the system.

An additional argument that nuclear motion may limit the rate of energy transfer from B* to P comes from comparing the initial absorbance difference spectrum of B* in R-26 and in the mutant lacking P (Figure 4). The spectrum of the bleaching due to B* in the mutant is broader (19 nm fwhm) than that of R-26 reaction centers (15−17 nm fwhm depending on whether or not P is oxidized). This is the opposite of what is observed in the ground-state spectrum where VR(L157) has a narrower spectrum than either the oxidized or unoxidized R-26 reaction centers (Figure 1). It is likely that in the case of the mutant lacking P's Q_Y transition, where B* lives for a long time, that B* relaxes vibrationally, and then its stimulated emission shifts toward the lower energy side of the ground-state band of B. This results in an additional apparent absorbance decrease near 801 nm (the peak of the emission, Figure 6). This red-shifted stimulated emission is not observed in either oxidized or unoxidized R-26 reaction centers because the energy transfer occurs on the same time scale as the dynamic Stokes shift and in fact may be rate limited by the nuclear motion involved in the dynamic Stokes shift. The stimulated emission from unrelaxed B* is superimposed on the ground-state absorbance band in R-26 reaction centers and thus does not contribute to the spectral width of the absorbance change in this region. Interestingly, in the VR(L157) mutant, the peak of the initial absorbance decrease near 796 nm shifts toward lower energy by about 2 nm in the first 100−200 fs (Figure 3). This is consistent with a shift in the stimulated emission as the excited state relaxes vibrationally, though coherent events could also be responsible for the spectral changes at early times in the region of the excitation pulse.

The Mechanism of Energy Transfer When P Is Oxidized. If one accepts the idea that electron exchange is the dominant interaction between B* and P^+ that results in rapid quenching of B's excited state, then there are at least two types of mechanisms by which this exchange could be accomplished. One possibility is simply that a concerted exchange reaction occurs, resulting in an excited singlet state of P^+,P^{+}*, which then decays to P^+. One could test this by making ultrafast absorbance measurements in the 1260 nm region where P^+ (but presumably not P^{+}*) absorbs. Another possibility is that the

electron exchange occurs by two sequential reactions with B* initially donating an electron to P^+. One version of this reaction involves initial electron transfer forming the intermediate state B^+P (transfer from the LUMO of B* to the HOMO of P^+), which then undergoes a second electron transfer from P to B^+, forming the ground state, P^+B. However, this should result in a transient recovery of ground-state P and an associated absorbance increase near 865 nm, which would then decay on a fast time scale, re-forming P^+. This was not observed even on a very rapid time scale (Figure 3), though the presence of some long-lived bleaching in this region (presumably due to a small concentration of unoxidized reaction centers, as described above) could have made detection of a small amount of the B^+P state difficult. Another possible intermediate in the decay of B^*P^+ by sequential electron transfer is B^+P^* (transfer from the LUMO of B* to the LUMO of P^+), which then undergoes a second electron transfer forming either BP^+ or BP^{+*}. Formation of B^+P^* should be detectable by performing transient absorbance change measurements in the 1260 nm band of P^+.

The Mechanism of B* and H* Decay in the Absence of P. The results from VR(L157) reaction centers also speak to another interesting issue. In a previous report we considered the possibility that charge separation in R-26 reaction centers could occur directly from B* and/or H* forming charge-separated states such as B^+H^-. Others have also considered B^+H^- as a possible intermediate in electron transfer.[42−44] In the case of B* decay following 800 nm excitation of the VR-(L157) mutant reaction centers, it is clear that if B^+H^- is a decay intermediate, either the rate of its formation is very slow or its equilibrium population is very small since B* lives for hundreds of picoseconds, and there is no spectral evidence for charge separation (Figures 2 lower panel, 3 lower panel, 7). In the case of H* decay following 760 nm excitation, most of the energy is transferred to B* on a 100 fs or faster time scale, similar to that observed in reaction centers containing P (Figures 2 upper panel and 4). However, one cannot rule out the possibility that some H^- is formed during this process. There is residual bleaching many picoseconds after 760 nm excitation in the VR(L157) mutant reaction centers, and this could represent a small yield of B^+H^- (Figure 4, upper panel), but this apparently is not a major product of H* decay. It is also possible that the spectral changes near 760 nm after the first few hundred femtoseconds represent a vibrationally hot ground state of H formed after energy transfer from H* to B.

The lack of charge separation from B* (and most likely H*) in VR(L157) raises some interesting questions regarding the nature of the cation and anion states of B and H. One might think that charge separation directly from B* would be energetically and kinetically possible. Comparing the absorbance and emission spectra of B and P, B* should be about 170 meV higher in energy than P*, placing B* at about 1.56 eV. It is already known that electron transfer can take place from P*, forming $P^+H_A^-$ in mutants with a P/ P^+ oxidation potential 260 mV above wild-type.[45] The oxidation potential of bacteriochlorophyll in solution is only about 200 mV higher than that of P.[46] Given this and the extra energy in B* compared to P*, one would think that B^+H^- formation would be thermodynamically possible from B*. In addition, ENDOR studies done on a mutant in which one of the bacteriochlorophylls in the special pair has been replaced by a bacteriopheophytin (a heterodimer mutant, see, for example, ref 47) show that during charge separation practically all of the electron hole resides on the bacteriochlorophyll of the heterodimer.[48,49] In this mutant, the heterodimer is similar in many respects to a monomeric bacteriochlorophyll but still undergoes charge separation on the picosecond time scale.[50] B* in VR(L157) reaction centers has a lifetime on the order of nanoseconds, providing ample time for a thermodynamically favorable reaction to occur.

However, no charge separation is apparent in VR(L157) in any of the measurements done here, implying that some thermodynamic or kinetic barrier does exist. It is probable that the protein electrostatics play a major role in making B^+H^- unfavorable. Electrostatic modeling of solubilized *Rhodopseudomonas viridis* reaction centers surrounded by water (assuming a dielectric constant of 2 for the protein) suggests that the environment of P is about 230 mV more favorable for stabilizing a positive charge than the environment of B_A, making $B_A^+H_A^-$ formation considerably less favorable than $P^+H_A^-$ formation.[51] The same study also suggests that $B_B^+H_B^-$ should be about 140 mV less favorable (on purely electrostatic grounds) than $P^+H_A^-$. Further work will be required to understand the origin of the lack of charge separation or excited-state quenching mechanisms in the VR(L157) mutant.

Conclusions

This report shows that the special pair, in either the oxidized or moderate potential form, is necessary and sufficient to rapidly quench B*. The energy transfer from B to P is probably not rate limited by the donor−acceptor coupling, and the rate may be limited by nuclear movement. In addition, no charge separation on any time scale is observed in a mutant which lacks P. Instead, B* appears to be very long-lived.

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