B-Side Electron Transfer in a *Rhodobacter sphaeroides* Reaction Center Mutant in Which the B-Side Monomer Bacteriochlorophyll Is Replaced with Bacteriopheophytin

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The mutation, H(M182)L, in the *Rhodobacter sphaeroides* reaction center (RC) results in the replacement of the monomer bacteriochlorophyll on the inactive side (B-side) of the RC with a bacteriopheophytin (the new cofactor is referred to as ϕ_B). In ϕ_B containing RCs, P* stimulated emission decays with an accelerated time constant of 2.6 ± 0.1 ps at room temperature compared to 3.1 ± 0.2 ps in WT RCs. Analysis of the time-resolved spectra implies that two states are being formed during the initial reaction in the mutant: the usual $P^+H_A^-$ state, as seen in WT, and a new state, $P^+\phi_B^-$. $P^+\phi_B^-$ is formed during the decay of P* and recombines to the ground state with a lifetime of 200 ± 20 ps. The yield of the $P^+\phi_B^-$ state is 35 ± 5% at room temperature, while the remaining 65 ± 5% of the initial electron-transfer results in $P^+H_A^-$. There does not appear to be any further electron transfer from ϕ_B^- to H_B. Apparently, in the H(M182)L mutant, the state $P^+\phi_B^-$ is lower in free energy than the $P^+H_B^-$ state.

Introduction

The structure and function of photosynthetic reaction centers from purple nonsulfur photosynthetic bacteria such as *Rhodobacter (Rb.) sphaeroides* have been studied extensively.[1−8] The reaction center (RC) is a pigment−protein complex consisting of three protein subunits L, M, and H, which coordinate 10 cofactors: 4 bacteriochlorophylls (BChl), 2 bacteriopheophytins (BPh), 2 quinone molecules, a carotenoid molecule and a nonheme iron (see Figure 1 for details).

One of the most remarkable aspects of RC structure is the near-rotational symmetry of the BChl, BPh, and quinone cofactors. Because of this symmetry, there are two potential pathways for photosynthetic electron transfer (labeled A and B in Figure 1), both starting with the initial electron donor, P (a pair of bacteriochlorophylls), and then proceeding through a monomer bacteriochlorophyll (either B_A or B_B) and a bacteriopheophytin (either H_A or H_B) to a ubiquinone (either Q_A or Q_B). However, initial electron transfer occurs almost exclusively along the cofactors on the A-side in wild-type (WT) RCs.[9−13] Upon excitation of P with light, an electron is transferred to H_A (presumably via B_A) in about 3 ps. Subsequently, the electron is transferred to the primary quinone acceptor, Q_A, in about 200 ps and then to the secondary quinone acceptor, Q_B, in about 200 μs. The quantum yield of this process is near unity.[14]

Electron transfer along the B-side was observed in the double mutant G(M201)D/L(M212)H of *Rb. capsulatus*. In this particular mutant, the bacteriopheophytin electron acceptor, H_A, is replaced with bacteriochlorophyll making it possible to see small changes in the Q_X transition region of H_B. The second mutation, G(M201)D modifies the environment of B_A. As a consequence, charge separation along the B-side cofactors was observed with the quantum yield of 15%.[15] These results suggested that the directionality of electron transfer is largely determined by the relative energies of $P^+B_A^-$ and $P^+B_B^-$. Theoretical calculations of radical pair energies using the crystal structure of the RC from *Rhodopseudomonas (Rp.) viridis* resulted in an estimate of the free energy of $P^+B_A^-$ that was slightly below that of P*, while $P^+B_B^-$ was calculated to be about 23 kJ mol^{-1} (0.24 eV) higher in energy than P*.[16] If these estimates are accurate, one would expect a strong preference for charge separation along the A-side cofactors, as is observed.

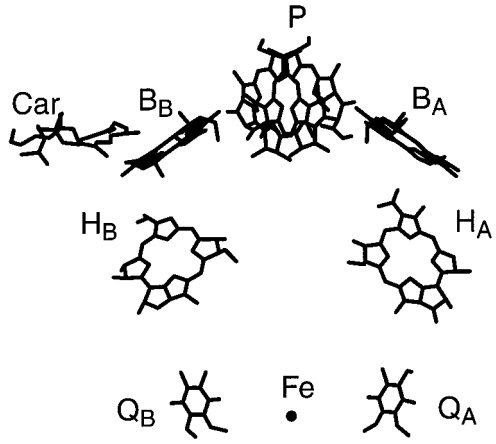

Figure 1. Crystal structure of the cofactors from the *Rb. sphaeroides* reaction center. P denotes a pair of bacteriochlorophylls; B_A and B_B, monomer bacteriochlorophylls; H_A and H_B, bacteriopheophytins; and Q_A and Q_B, ubiquinones. Car stands for the carotenoid molecule, Fe for the nonheme iron atom. The carotenoid and the side chains of the pigments are truncated.

In this report, the electron-transfer properties of a RC mutant of *Rb. sphaeroides*, H(M182)L, is investigated. In this mutant the histidine (H) ligand of B_B at position M182 has been changed to a leucine (L), and, as a consequence, a bacteriopheophytin (referred to as ϕ_B) is incorporated in place of B_B. One would expect that the $P^+\phi_B^-$ state would be considerably lower in energy that $P^+B_B^-$ (due to the lower midpoint potential of BPh compared to BChl[17]), thus enhancing the probability of initial B-side electron transfer.

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Materials and Methods

The codon for histidine at the position M182 (CAC) was replaced by TTG which encodes leucine. The mutagenesis was performed as previously described.[18] RCs were isolated from semiaerobically grown *Rb. sphaeroides* according to previously published procedures.[18,19] For spectroscopic measurements, RCs were resuspended in either 15 mM Tris-HCl (pH 8.0), 0.025% LDAO, and 1 mM EDTA (unreduced samples) or 15 mM Tris-HCl (pH 8.0), 0.05% Triton X-100, and 1 mM EDTA (reduced samples). The addition of 100 μM of *o*-phenanthroline was used to block Q_A to Q_B electron transfer. To block the electron transfer from H_A to Q_A, either 1 mM sodium dithionite was used to chemically reduce Q_A or removal of Q_A using 4% LDAO buffer with *o*-phenanthroline was performed.[20] The BChl to BPh ratio of the WT and H(M182)L mutant RCs was analyzed by performing a pigment extraction in a 7:2 (v/v) acetone−methanol solution[21] and analyzing the extract using HPLC.[22] The BChl:BPh ratio for the H(M182)L mutant RCs was calculated from the ratio of the HPLC peaks' areas after normalizing with the assumption that the ratio in WT RCs is 2.

Steady-state absorption spectra were measured on a Cary-5 spectrophotometer (Varian). Transient absorption measurements with subpicosecond time resolution were performed on a spectrometer based on a Clark-MXR laser system.[23] For excitation of the sample, 860 nm light pulses of about 150 fs duration (fwhm) were produced in an optical parametric amplifier. The pulses were filtered through an 860 nm interference filter with a 10 nm spectral width (Melles−Griot) and polarized at the magic angle (54.7°) with respect to the probe light using sheet polarizers (Melles−Griot). The transient absorption spectra were measured in overlapping spectral regions with 2 nm spectral resolution over either a 20 ps time interval (0.2 ps delay increments) or a 1 ns time interval (10 ps increments).

Measurements of RCs with intact Q_A were performed in a rotating circular cell with a 2 mm optical path which removed the sample completely from the excitation region between pulses. The samples with reduced or removed Q_A were measured in an airtight, 2 mm path length glass cuvette with constant stirring. All measurements were performed at room temperature. The OD of the samples was about 1.2 at 800 nm. Data were analyzed using a locally written global analysis routine based on Matlab 5.1 software (Mathworks). Transient absorption kinetics were fitted to the sum of exponential terms convoluted with the instrument response profile which was approximated by a Gaussian function. Zero delay and dispersion corrections were determined from measurements of CS_2 birefringence[24] and data were empirically corrected as previously described.[25]

Results and Discussion

The ground-state absorption spectra of the WT and the H(M182)L mutant RCs at room temperature are shown in Figure 2. Evidently, the spectral band, which peaks at 802 nm in WT RCs, has blue-shifted to 797 nm in the mutant. In addition, the Q_X band of the BChls at 600 nm has become reduced, whereas the Q_X band of the BPhs near 540 nm has gained oscillator strength. These spectral changes are consistent with the replacement of B_B with a BPh in the mutant. Further evidence for this is provided by pigment extraction and HPLC analysis (see Methods) which resulted in a BChl:BPh ratio of 1.07 ± 0.05 in the H(M182)L mutant RCs. The difference between the H(M182)L and WT ground-state absorption spectra (inset in Figure 2) shows that the new Bph, ϕ_B, has transitions near 785 and 540 nm (there are positive bands at these wavelengths in the difference spectrum). It is interesting to note that the newly incorporated BPh does not show the same spectral properties as H_A or H_B, i.e., its Q_Y band maximum is red-shifted relative to the maximum of the native H band (760 nm). The mutation, H(M182)L, in *Rb. capsulatus* RCs was also shown to result in replacement of B_B with BPh.[22,26] Again, the new BPh in this mutant did not absorb at 760 nm like the other RC BPh molecules, but rather near 785 nm. A similar effect was noted in the β mutant, in which H_A is replaced with BChl.[27] In this case, the spectral properties of the new BChl did not correspond to the properties of B_A and B_B. All of these effects can be attributed to the different local pigment−protein interactions, which modify the spectral properties of the pigments.

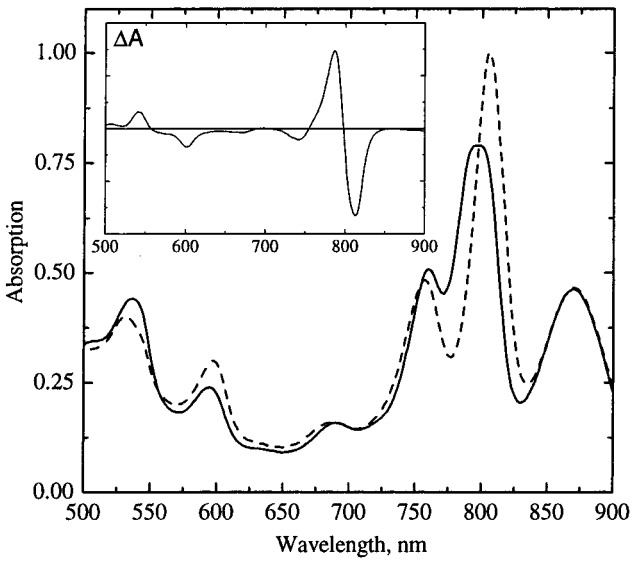

Figure 2. Ground-state absorption spectra of H(M182)L mutant (solid line) and WT (dashed line) RCs at room temperature. Spectra were normalized at the maximum of the P band (865 nm). The inset shows the difference between the H(M182)L and WT spectra.

Transient absorption measurements with subpicosecond time resolution were performed on samples with intact Q_A as well as samples with removed or chemically reduced Q_A. In all cases, it was found that the P* stimulated emission decay in the H(M182)L mutant RCs is substantially faster than that in WT RCs. From single-exponential fits of the kinetics at 921 nm, lifetimes of P* stimulated emission decay were determined to be 2.6 ± 0.1 ps and 3.1 ± 0.2 ps for H(M182)L and WT RCs, respectively. This indicates that either the primary charge separation rate is altered in the mutant or that there are pathways for P* decay in addition to electron transfer to H_A.

Subsequent electron-transfer reactions on the several hundred picosecond time scale were investigated in samples with intact Q_A. It was determined that the bleaching of the H band at 760 nm in the H(M182)L mutant RCs decays with a lifetime of 230 ± 10 ps, in good agreement with the 210 ± 10 ps time constant found in WT RCs. However, from the decay of the bleaching at 865 nm it was determined that there is a significant ground-state recovery of P in the mutant (about 30−40%) which also occurs with a roughly 200 ps decay time. It should be noted, that in WT RCs there is almost no loss of the 865 nm bleaching observed on the 200 ps time scale during electron transfer from H_A to Q_A.[1]

The decay-associated spectrum of the 230 ps component in the H(M182)L mutant displayed a pronounced bleaching centered at 787 nm, indicating that ϕ_B was involved in the 230 ps transient state(s). It seems likely that some state involving ϕ_B is present during the same time as $P^+H_A^-$ and decays with roughly the same time constant as the $P^+H_A^-$ to $P^+Q_A^-$ reaction.

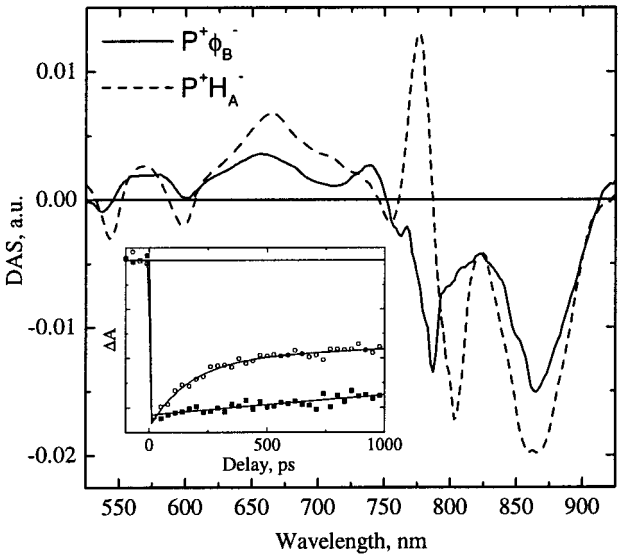

Figure 3. Decay-associated spectra corresponding to the charge separated states $P^+\phi_B^-$ (solid line) and $P^+H_A^-$ (dashed line) in quinone-removed RCs from H(M182)L. The spectra were determined from the global fitting of 1 ns time-scale transient absorption data with the sum of two exponential functions. The inset shows the transient absorption kinetics measured at 865 nm in RCs from the H(M182)L mutant (open circles) and WT (black squares) at room temperature. For clarity, only every third data point is shown. Solid lines represent fits using a two-exponential function with the lifetimes of 200 ps (amplitude 40%) and 10 ns (60%) in case of the H(M182)L mutant and one-exponential function with the lifetime of 10 ns in case of WT.

To distinguish between $P^+H_A^-$ and the state involving ϕ_B, samples with removed or chemically reduced Q_A were investigated. Upon quinone removal or reduction in WT RCs, the transient spectrum of the $P^+H_A^-$ state is formed within a few picoseconds after excitation and remains stable for 10 ns or more at room temperature.[1,7] However, in the H(M182)L mutant, even after quinone removal or reduction, a 30−40% ground-state recovery of P (relative to the bleaching at 10 ps) with a lifetime of 200 ± 20 ps is observed in the 865 nm band (see inset in Figure 3). The remaining 60−70% of the early time ground-state bleaching of P corresponds to a long-lived (greater that 1 ns) kinetic component which has spectrum similar to $P^+H_A^-$ in WT RCs (see Figure 3). Apparently, the decay of P* in the mutant results in two states: $P^+H_A^-$ (60−70%) and a new state involving ϕ_B as indicated by the bleaching near 787 nm which decays on the 200 ps time scale.

One likely possibility for the new state is $P^+\phi_B^-$. This hypothesis is supported by measurements in the other spectral regions. Absorbance changes decaying with the same lifetime (200 ± 20 ps) were detected at 538 nm (BPh Q_X ground-state region), 600 nm (BChl Q_X ground-state region) and near 655 nm (BPh anion-absorbance region) (see Figure 3). Bleaching near 538 nm is consistent with the idea that ϕ_B is involved in the 200 ps state, since H_A and H_B have been shown to absorb at 545 and 530 nm, respectively,[1] and the difference between the ground-state absorbance spectra of H(M182)L and WT showed a new peak at about 540 nm (Figure 2, inset). The decay of the bleaching at 600 nm correlates with the decay of the bleaching at 865 nm, again indicating ground-state recovery of P. The broad band at around 655 nm in the decay-associated spectrum of the 200 ps component indicates that the 200 ps state involves the decay of a BPh anion.[27] All of these spectral characteristics of the transient state are consistent with the picosecond formation of a $P^+\phi_B^-$ state followed by recombination with a lifetime of 200 ± 20 ps.

The yield of the $P^+\phi_B^-$ state (compared to the overall yield of charge separation, which appears to be similar to the yield in WT) is apparently 30−40%. Therefore, H(M182)L mutant RCs represent an excellent system for studying B-side electron transfer. The situation is made even better by the fact that the $P^+\phi_B^-$ state lives for 200 ps in quinone removed or reduced RCs: 2 orders of magnitude longer than the initial charge separation reaction and 2 orders of magnitude shorter than the decay time of $P^+H_A^-$. This makes the new B-side charge separated state particularly easy to detect and quantitate.

It is noteworthy that electron transfer does not appear to continue on from $P^+\phi_B^-$ forming $P^+H_B^-$ in this mutant. Other mutants which enhance B-side electron transfer have resulted in transfer at least as far as H_B,[15] and multiphoton excitation of R-26 reaction centers also results in roughly 30% electron transfer along the B-side as far as H_B.[28] The lack of electron transfer past $P^+\phi_B^-$ in the H(M182)L mutant is presumably due to the energetics of the $P^+\phi_B^-$ state. As determined in vitro, the reduction potential of BPh is about 300 mV lower than that of BChl.[17] Therefore, one can assume, that due to the replacement of B_B with ϕ_B, the state $P^+\phi_B^-$ in the mutant is lower in energy than $P^+B_B^-$ in WT by roughly a few hundred meV. As mentioned above, theoretical calculations using the *Rp. viridis* RC crystal structure suggested that the $P^+B_B^-$ state was ~0.24 eV higher than P*.[16] It is thus reasonable that in the H(M182)L mutant the state $P^+\phi_B^-$ may be lower in free energy than P* and result in favorable electron transfer to ϕ_B with a relatively high yield. However, it is surprising that the energy of $P^+\phi_B^-$ would be lower than the energy of the state $P^+H_B^-$, which, from experiments such as those mentioned above,[15,28] is presumably significantly below P*. In any case, electron-transfer stops at ϕ_B in the H(M182)L mutant, and later decays by recombination to the ground state, rather than undergoing further electron transfer down the B-side of cofactors. To further explore the nature of the activation barriers that control relative electron transfer rates along the two sides in this mutant, low-temperature kinetic measurements are being performed.

The spectral details of the difference absorbance spectrum of $P^+\phi_B^-$ (see Figure 3) are also of interest. Like the state $P^+B_A^-$, determined at low temperature in RCs with plant pheophytin in place of the bacteriopheophytins,[29] $P^+\phi_B^-$ appears to lack almost entirely the electrochromic shift of the B-band which is normally associated with P^+ formation (see for example the $P^+H_A^-$ spectrum in Figure 3).[7] This is partly due to the fact that there is only the B_A absorbance transition to shift in the $P^+\phi_B^-$ state, since the ϕ_B transition is bleached. In addition, the close proximity of P^+ and ϕ_B^- may decrease the electric field strength felt by B_A and thus decrease the magnitude of its electrochromic shift.

As already mentioned, the mutation H(M182)L in *Rb. capsulatus* RC also results in replacement of B_B with the BPh.[22,26,30] In the *Rb. capsulatus* mutant, preliminary transient absorbance measurements indicated that some B-side electron transfer might be occurring, but the preparations were heterogeneous and consistent results were difficult to obtain.[26]

Interestingly, the H(M182)L mutant reaction center's pigment composition is apparently the same as the native composition of *Chloroflexus (Cf.) aurantiacus* RCs, which also have three BChls and three BPhs. The structure of the *Cf. aurantiacus* RC is thought to be similar to that of *Rb. sphaeroides*, as estimated from protein sequence comparisons and relative orientations of the pigments, except that the monomer BChl on the B-side in WT *Rb. sphaeroides* is replaced by BPh in *Cf. aurantiacus*. However, there is no evidence for the B-side electron transfer

in *Cf. aurantiacus* RCs.[31] Apparently, the environment of the binding pocket for ϕ_B and for the BPh in the analogous position in *Cf. aurantiacus* RCs is not the same. This is also suggested by the ground-state spectrum of *Cf. aurantiacus* RCs, which shows that all three BPhs absorb at 760 nm. One can only speculate that because of the differences in the binding pockets, the B-side electron transfer in *Cf. aurantiacus* RCs is unfavorable. This would imply a dramatic destabilization of the BPh anion in *Cf. aurantiacus* RCs relative to ϕ_B^- in *Rb. sphaeroides* H(M182)L RCs.

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