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The effect of exchange of bacteriopheophytin *a* with plant pheophytin *a* on charge separation in Y(M210)W mutant reaction centers of *Rhodobacter sphaeroides* at low temperature

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Abstract

The bacteriopheophytin *a* molecules at the H_A and H_B binding sites of reaction centers (RCs) of the Y(M210)W mutant of *Rhodobacter sphaeroides* were chemically exchanged with plant pheophytin *a*. The Y(M210)W mutation slows down the formation of H_A^-, presumably by raising the free energy level of the $P^+B_A^-$ state above that of P* due to increasing the oxidation potential of the primary electron donor P and lowering the reduction potential of the accessory bacteriochlorophyll B_A. Exchange of the bacteriopheophytins with pheophytin *a* on the contrary lowers the redox potential of H_A, inhibiting its reduction. A combination of the mutation and pigment exchange was therefore expected to make the A-side of the RC incapable of electron transfer and cause the excited state P* to deactivate directly to the ground state or through the B-side, or both. Time-resolved absorption difference spectroscopy at 10 K on the RCs that were modified in this way showed a lifetime of P* lengthened to about 500 ps as compared to about 200 ps measured in the original Y(M210)W RCs. We show that the decay of P* in the pheophytin-exchanged preparations is accompanied by both return to the ground state and formation of a new charge-separated state, the absorption difference spectrum of which is characterized by bleachings at 811 and 890 nm. This latter state was formed with a time constant of ca. 1.7 ns and a yield of about 30%, and lasted a few nanoseconds. On the basis of spectroscopic observations these bands at 811 and 890 nm are tentatively attributed to the presence of the $P^+B_B^-$ state, where B_B is the accessory bacteriochlorophyll in the "inactive" B-branch of the cofactors. The B_B molecules in Y(M210)W RCs are suggested to be spectrally heterogeneous, absorbing in the Q_y region at 813 or 806 nm. The results are discussed in terms of perturbation of the free energy level of the $P^+B_B^-$ state and absorption properties of the B_B bacteriochlorophyll in the mutant RCs due to a long-range effect of the Y(M210)W mutation on the protein environment of the B_B binding pocket.

Keywords: Purple bacterium; *Rhodobacter sphaeroides*; Reaction center; Mutation; Pigment exchange; Bacteriopheophytin; Pheophytin; Charge separation

1. Introduction

One of the remarkable and least understood properties of the reaction center (RC) of purple bacteria is the strong asymmetry of its photochemical properties as opposed to the near C_2 symmetry of the cofactor and global protein structures. X-ray crystallographic analysis has revealed that the RC cofactors are arranged in two symmetry positioned branches (denoted A and B), which constitute two possible ways for photosynthetic electron transfer [1,2]. Both ways start with the primary electron donor (P, a bacteriochlor-

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Abbreviations: $B_{A,B}$, bacteriochlorophyll located at the A- or B-branch, respectively; BChl, bacteriochlorophyll; BPheo, bacteriopheophytin; $H_{A,B}$, bacteriopheophytin located at the A- or B-branch, respectively; P, dimeric BChl primary electron donor; Pheo, pheophytin; $Pheo_{A,B}$, pheophytin replacing $H_{A,B}$; $Q_{A,B}$, acceptor quinones; RC, reaction center

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ophyll (BChl) dimer), and proceed via a monomeric BChl molecule (B_A or B_B) and a bacteriopheophytin (BPheo) molecule (H_A or H_B) to a quinone acceptor (Q_A or Q_B). Under normal conditions all of the photo-induced electron transfer appears to occur along the A-branch. Upon excitation of P an electron is transferred from the excited singlet state P* to H_A with a time constant of ~ 3 ps at room temperature and ~ 1.5 ps at cryogenic temperatures, forming the charge-separated state $P^+H_A^-$. Then H_A^- transfers an electron to Q_A with a time constant of ~ 200 ps at room temperature and ~ 100 ps at 77 K and below, yielding the state $P^+Q_A^-$. Within experimental uncertainty, the overall quantum yield of this charge-separation process is unity both at room and at cryogenic temperatures (for reviews, see Refs. [3–5]). Time-resolved spectroscopic studies have provided compelling evidence that primary charge separation in bacterial RCs is a two-step process in which an electron is transferred to B_A prior to H_A, the electron transfer rate from B_A^- to H_A being several times faster than that from P* to B_A [6–9]. Recent measurements with selective excitation of P by femtosecond pulses (less than 30 fs) presented a new tool for the study of the electron-nuclear coupling during primary charge separation between P* and B_A [10–16]. According to this study the electron from P* can reach B_A during first 120 fs after excitation of P [14–16].

Various attempts have been made to enhance the rate of electron transfer via the B-branch, either by making conditions for the A-branch transfer less favorable [17] or by facilitating B-chain electron transfer [18] or by a combination of both [19,20]. Significant electron transfer along the B-branch was observed in RCs of the double mutant G(M201)D/L(M212)H of *Rhodobacter (Rba.) capsulatus* [17]. The L(M212)H mutation causes a BChl, denoted β, to be incorporated in place of the native $BPheo_A$ (β-type mutant). Due to the difference in the redox properties of the pigments (BChl is more difficult to reduce than BPheo in vitro) the state $P^+\beta^-$ in the mutant RCs was proposed to be at higher free energy than $P^+H_A^-$ in wild-type RCs. A perturbation of the protein environment of B_A due to the G(M201)D mutation was expected to increase the free energy of $P^+B_A^-$. As a consequence, P* had a longer lifetime in the double mutant RCs (15 ps) than in wild-type RCs (~ 4 ps), and its decay was accompanied by electron transfer to H_B with a yield of the $P^+H_B^-$ state of 15%. Similar A-branch mutations of *Rhodobacter sphaeroides* designed to prolong the lifetime of P* have been described [21]; they did not, however, give rise to appreciable B-chain electron transfer. Adding to the double mutant G(M201)D/L(M212)H of *Rba. capsulatus* a third mutation, S(L178)K, that was supposed to lower the free energy of $P^+B_B^-$ due to alteration of the environment near B_B, increased the yield of $P^+H_B^-$ to 23% [19]. Slightly higher (~ 30%) yield of $P^+H_B^-$ has been reported for a β-type mutant of *Rba. capsulatus* in which the free energy of $P^+B_A^-$ was raised and the free energy of $P^+B_B^-$ simultaneously lowered by swapping a phenylalanine for a tyrosine near B_B (L181) and a tyrosine for a phenylalanine near B_A (M208) [20]. Recently a mutation H(M182)L has been introduced into *Rba. sphaeroides* that has resulted in the incorporation of a BPheo molecule, labeled ϕ_B, into the B_B binding site [18]. The ϕ_B containing RCs showed 30–40% yield of the $P^+\phi_B^-$ state. No electron transfer was observed from ϕ_B^- to H_B, and $P^+\phi_B^-$ was postulated to be lower in free energy than $P^+B_B^-$, and even than $P^+H_B^-$ [18]. Combining the ϕ_B, β and G(M203)D mutations with the A(M260)W mutation (which inhibits Q_A binding) an efficiency of 35–45% of B-branch electron transport was achieved in the quadruple mutant RCs of *Rba. sphaeroides* [22].

All these findings demonstrate that the normally inactive B-branch can be made accessible for electron transfer by changing the free energy balance between the A- and B-side charge-separated states due to specific mutations in the RC. In one case [23] an effective, nearly 40% B-side electron transfer with formation of the $P^+H_B^-$ state has been observed in native *Rba. sphaeroides* R-26 RCs upon high-intensity excitation. The mechanism of this process is not clear, but, most probably, it involves electron transfer between simultaneously generated excited states P* and B_B* [23]. Hartwich et al. [24] have reported that B-branch electron transfer (H_B bleaching at 533 nm) could be achieved by chemical exchange of the BChl at the B_A site with vinyl-bacteriochlorophyll *a*, which has in solution a reduction potential ~ 150 mV higher than the native BChl.

In this communication we report the results of low-temperature (10 K) steady-state and time-resolved experiments on modified RCs of *Rba. sphaeroides* in which the states $P^+B_A^-$ and $P^+H_A^-$ were energetically raised by a combination of mutation and pigment exchange. The tyrosine at position M210 (the analog of M208 in *Rba. capsulatus*) was mutated to a tryptophan and in the resulting Y(M210)W mutant RCs, both BPheos, H_A and H_B, were exchanged with plant pheophytin *a* (Pheo). It has been previously shown that replacing the tyrosine M210 by a tryptophan slows down electron transfer from P* to H_A, presumably by raising the free energy level of $P^+B_A^-$ above that of P* [25–27]. Exchange of the BPheo *a* molecules at the H_A and H_B binding sites by Pheo *a*, which in solution has substantially more negative reduction potential than BPheo, was shown to increase the free energy of $P^+Pheo_A^-$ in *Rba. sphaeroides* R-26 RCs [7,9,28–30], with dramatic increase of the lifetime of B_A^- at low temperature [9]. We present evidence that the combination of both changes brings about an inhibition of electron transfer in the A-branch, with an increase of the lifetime of P*. Our data indicate that P* decay is accompanied by formation of a new charge-separated state in about 30% of the modified RCs. On the basis of spectroscopic observations this state is tentatively assigned to $P^+B_B^-$.

2. Materials and methods

The construction of the site-directed mutant Y(M210)W from the deletion mutant *Rba. sphaeroides* 2.4.1. (ΔLM1) and subsequent isolation and purification of the mutant RCs were performed as described previously [26]. Mutant RCs containing plant pheophytin *a* instead of the native bacteriopheophytin *a* molecules were prepared using the procedure developed by Scheer et al. [31,32] for RCs of *Rba. sphaeroides* R-26 with a slight modification according to Franken et al. [33]. Room-temperature measurements of the decay kinetics of the bleaching of P at about 865 nm (excitation at 532 nm) showed that more than 95% of the secondary acceptor quinone Q_B was lost from the mutant RCs during the exchange procedure. This was concluded from the fact that, in contrast to the original Y(M210)W RCs, which displayed a decay on the time scale of seconds (expected for $P^+Q_B^-$ charge recombination), the bleaching in the pheophytin-exchanged preparations decayed on the time scale of hundreds of milliseconds, characteristic of charge recombination between P^+ and Q_A^-. Borohydride treatment of Y(M210)W mutant RCs was done as described earlier for *Rba. sphaeroides* R-26 RCs [34,35]. All RCs used in this study were suspended in 10 mM Tris–HCl (pH 8.0) buffer containing 0.1% lauryldimethylamine *N*-oxide (LDAO). To obtain a clear glass at low temperature, the samples were mixed with glycerol (66% v/v).

Low-temperature time-resolved absorption difference measurements were performed with a home-built amplified dye laser system, operating at 10 Hz, as described earlier [9,36]. Excitation pulses were obtained by amplifying the white-light continuum in a dye cell (LDS 867, Exciton). Wavelengths shorter than 850 nm in the pump beam were cut off with an RG850 filter (Melles Griot). The intensity was adjusted to provide a maximum bleaching of less than 10% of the long-wavelength P absorption band. The time resolution was about 600 fs, and the accuracy of the wavelength calibration was ± 1 nm. Pump and probe pulses were polarized at the magic angle with respect to each other.

Absorption difference spectra were routinely measured in a time interval of 1.9 ns. In a separate experiment, the delay line of the described spectrophotometer was lengthened in a way to measure the difference spectrum in pheophytin-modified RCs at 10 ns. Due to the strong difference in the intensity of the pump beam, the spectrum taken at 10 ns could only be compared to the 1.9-ns spectrum in shape but not in amplitude.

At every delay time, a combination of two spectra each having a width of 155 nm, one starting from ~ 745 nm and another from ~ 845 nm, was used to cover the wavelength region from about 750 to about 1000 nm. The two spectra were combined by normalizing the amplitude of their long-wavelength P band bleaching. For clarity, part of the overlapping 745–900 nm spectrum is not plotted in the corresponding figures.

Measurements at 10 K were performed using a helium flow cryostat (Utrecs-LSO, Tartu, Estonia). The sample had an absorbance of 0.6–0.8 in the maximum of the long-wavelength band of P at low temperature; the optical pathway was 0.5 mm.

Flash-induced absorption changes in the millisecond range were measured as described by Franken and Amesz [37]. Excitation flashes were provided by a Q-switched frequency-doubled Nd:YAG laser (15 ns, 532 nm, 90 mJ per pulse).

Low-temperature steady-state absorption spectra were measured with a single-beam spectrophotometer using an Oxford Instruments flow cryostat as described by Otte [38]. The spectral resolution was 0.5 nm.

3. Results and discussion

The steady-state and dynamic properties of RCs of the Y(M210)W mutant of *Rba. sphaeroides* were compared with those of a Y(M210)W mutant in which the BPheo *a* molecules at the H_A and H_B binding sites were exchanged with Pheo *a*. In order to obtain more information on the absorption of the monomeric bacteriochlorophylls, steady-state absorption spectra of Y(M210)W and pheophytin-exchanged Y(M210)W mutant RCs treated with sodium borohydride ($NaBH_4$) will also be discussed. We shall in the following refer to the pheophytin-exchanged Y(M210)W mutant as Y(M210)W-Pheo and to the Y(M210)W and Y(M210)W-Pheo mutants treated with sodium borohydride as Y(M210)W-$NaBH_4$ and Y(M210)W-Pheo-$NaBH_4$, respectively.

3.1. Ground-state absorption spectra

Fig. 1 shows the low-temperature (10 K) absorption spectra of the Y(M210)W mutant RCs of *Rba. sphaeroides*

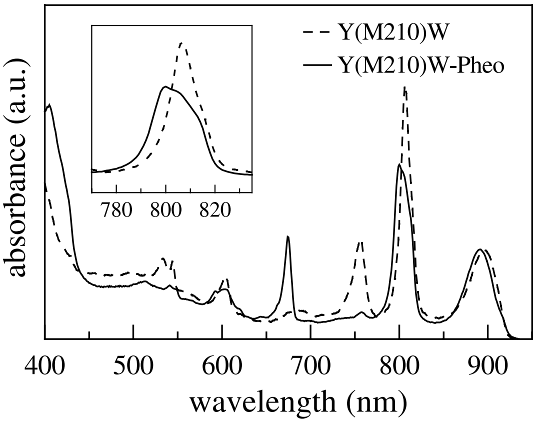

Fig. 1. Absorption spectra at 10 K of Y(M210)W RCs (dashed line) and Y(M210)W-Pheo RCs (solid line). The spectra were normalized at their P absorption band around 895 nm. The inset shows the accessory BChl Q_y spectral region in an expanded scale.

before (dashed line) and after (solid line) exchange of the BPheo *a* molecules with Pheo *a*. RCs of the Y(M210)W mutant show three main absorption bands in the Q_y region: around 895, 807 (with a shoulder at 815 nm) and 757 nm. This spectrum is virtually identical to those published earlier [25,26,39,40]. The long-wavelength band (895 nm) arises from the low-energy exciton transition of the primary electron donor P and the 807- and 815-nm bands can be ascribed to the two monomeric accessory BChls, B_A and B_B, respectively [5]. The high-energy exciton transition of P may also absorb in this spectral region [41,42]. The 757-nm band is assigned to the absorption by the BPheo molecules H_A and H_B.

Upon pigment exchange the BPheo band at 757 nm disappeared almost completely and the Q_y absorption band of the newly introduced Pheo *a* is located around 675 nm. These spectral changes indicate that the BPheo *a* molecules located at both the H_A and H_B binding sites were replaced by Pheo *a* with a very high yield. Comparing the absorption spectrum of Y(M210)W-Pheo RCs with similar spectra obtained for Pheo-exchanged RCs from *Rba. sphaeroides* R-26 [32,33], we estimate that in our preparation more than 95% of the total BPheos were exchanged. An exchange yield of about 95% was also determined by direct integration of the Q_y absorption band of BPheo in Y(M210)W RCs and of the remaining BPheo band in Y(M210)W-Pheo RCs. There is evidence that Pheo *a* is preferentially exchanged into the H_B binding site [33]. With the assumption of 100% H_B exchange, $\leq 10\%$ of the Y(M210)W-Pheo RCs still contain a BPheo molecule at the H_A site. This fraction of "H_B-only exchanged RCs" is expected to perform primary photochemistry that is essentially indistinguishable from that in the original Y(M210)W RCs.

The maximum of the long-wavelength band of P in the 10 K absorption spectrum of Y(M210)W-Pheo RCs was slightly blue-shifted compared to original Y(M210)W RCs (Fig. 1). The Q_y absorption band of the accessory BChls (B_A and B_B) was affected by pigment exchange more strongly: it broadened, decreased in amplitude and its maximum was blue-shifted by 8 to 799 nm. As a result, the accessory BChl absorption band was resolved into three components (derived from the second derivative), a maximum at 799 nm and two shoulders on the long-wavelength side at 806 and 813 nm, respectively (Fig. 1 and inset). Integration of the normalized absorption spectra of Y(M210)W RCs and Y(M210)W-Pheo RCs between 770 and 830 nm (Fig. 1, inset) indicated that the total dipole strength in this spectral region was virtually unaltered by pigment exchange. The 799-nm maximum in Y(M210)W-Pheo RCs appears to be due to a short-wavelength shift of the blue-most band (at 807 nm) of Y(M210)W RCs that is thought to arise principally from the Q_y transition of B_A [26]. A blue shift of the Q_y band of B_A to 799 nm has been reported earlier for the Pheo-exchanged RCs of *Rba. sphaeroides* R-26 [33]. A similar shift has also been observed in the absorption spectrum of the double FM197R/GM203D mutant RCs of *Rba. sphaeroides* [43]. In accordance with assigning the 799-nm band to B_A, the charge-separated state $P^+B_A^-$ in Pheo-exchanged RCs of *Rba. sphaeroides* R-26 at 5 K gave rise to a bleaching at 800 nm due to B_A reduction [9].

Genetic replacement of the accessory BChl at the B_B site by a BPheo molecule in the M182HL mutant RCs of *Rba. sphaeroides* has been shown to be accompanied by a disappearance of the shoulder at about 810 nm on the low-energy side of the 800-nm absorption band [44]. Instead, at 77 K a new feature was observed on the high-energy side of the 800-nm band. This finding indicates that the 810-nm shoulder seen in the low-temperature absorption spectrum of wild-type *Rba. sphaeroides* RCs is due to B_B, and that a contribution to this shoulder of the upper exciton band of P is very small. On the basis of these data, the shoulder at 813 nm in the low-temperature spectrum of Y(M210)W-Pheo RCs (as well as that at 815 nm in the original Y(M210)W mutant RCs) can be attributed to the Q_y transition of BChl at the B_B binding site.

In order to obtain more information on the nature of the 815-nm shoulder in the absorption spectrum of Y(M210)W and that at 813 nm in the spectrum of Y(M210)W-Pheo RCs, we performed experiments on RCs treated with sodium borohydride ($NaBH_4$). It has previously been reported that in *Rba. sphaeroides* R-26 RCs the contribution of the "inactive BChl" B_B to the ground-state absorption spectrum in the 810–815-nm region strongly decreased upon treatment with borohydride, without affecting the B_A absorption [34,35,45–47]. The primary electron transport in these chemically modified RCs was essentially unaffected, which indicates that the reaction with borohydride is limited to the inactive cofactor branch [45,46,48]. At present, there is no consensus on the mechanism of the $NaBH_4$ action. From spectroscopic and kinetic data it has been initially suggested that the B_B molecule is reduced by borohydride and then removed from the RC [34,45]. However, later Struck et al. [49] have reported that borohydride does not chemically change the pigment, but rather alters the interaction between the protein and the accessory BChl in the inactive branch, resulting in the shift of the B_B absorption from the 810–815-nm region to shorter wavelengths. No matter the mechanism, the selective effect of borohydride treatment on the absorption spectrum of the B_B molecule can be useful for determining the wavelength position of the Q_y band of B_B in the mutant RCs.

Fig. 2 (dashed line) shows the 10 K absorption spectrum of Y(M210)W-$NaBH_4$ RCs. Upon borohydride treatment the long-wavelength shoulder of the accessory BChl band at about 815 nm almost completely disappeared, leaving a single absorption peak at 807 nm (inset A, Fig. 2). The absorption around 759 nm increased compared to the non-treated Y(M210)W RCs, indicating that a new band appears in this region, which overlaps with the H_A and H_B absorption. Like in the borohydride-treated *Rba. sphaeroides* R-26 RCs [46], a shoulder near 780 nm was formed. In agreement with the results obtained on the wild-type *Rba. sphaeroides*

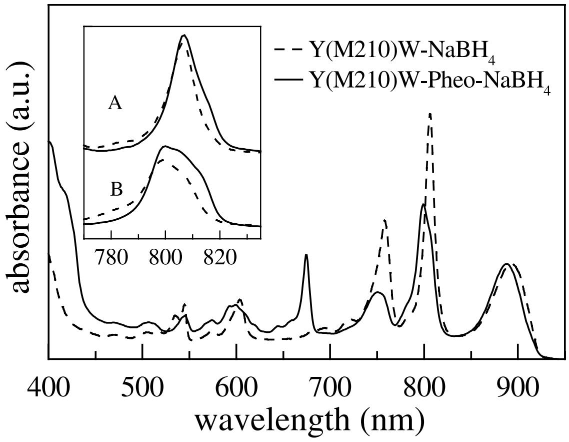

Fig. 2. Absorption spectra at 10 K of RCs treated with sodium borohydride: Y(M210)W-NaBH$_4$ RCs (dashed line) and Y(M210)W-Pheo-NaBH$_4$ RCs (solid line). Inset: Comparison of the absorption spectra in the accessory BChl region before (solid lines) and after (dashed lines) borohydride treatment. A: Y(M210)W and Y(M210)W-NaBH$_4$ RCs; B: Y(M210)W-Pheo and Y(M210)W-Pheo-NaBH$_4$ RCs. The spectra were normalized at their P absorption band.

RCs [50], borohydride treatment of Y(M210)W RCs was resulted in a strong decrease of the carotenoid absorption that suggests the loss of a considerable part of the RC carotenoids from the treated preparations.

The solid line in Fig. 2 shows the absorption spectrum of Y(M210)W-Pheo-NaBH$_4$ RCs. In this particular preparation about 25–30% of H$_B$-only exchanged RCs was estimated to be present. Again, borohydride treatment resulted in a strong decrease of the long-wavelength shoulder of the accessory BChl, at about 813 nm (inset B, Fig. 2). The band at 799 nm and the shoulder at 806 nm were also influenced by the treatment but to a significantly smaller extent. A new product band is now clearly visible around 747 nm and a shoulder at 780 nm is formed. Bound carotenoids were lost upon borohydride treatment as judged from the diminished carotenoid absorption in the Y(M210)W-Pheo-NaBH$_4$ RCs with respect to that in Y(M210)W-Pheo RCs.

In summary, the data presented allow one to conclude that the effect of borohydride treatment on the absorption band of the accessory BChls in the mutant RCs is essentially the same as in *Rba. sphaeroides* R-26 RCs: in both cases there is a strong decrease in absorption on the long-wavelength side of the Q$_y$ band of the B$_{A,B}$ molecules. By analogy to *Rba. sphaeroides* R-26 RCs we take this data as an indication that the shoulder at about 813–815 nm in the low-temperature spectra of Y(M210)W and Y(M210)W-Pheo RCs is due to absorption of the B$_B$ molecule. Thus we ascribe the band at 813 nm in the 10 K absorption spectrum of Y(M210)W-Pheo RCs to B$_B$, and that at 799 nm to B$_A$. This assignment corresponds to the generally accepted view that in *Rba. sphaeroides* RCs the blue-most absorbing accessory BChl Q$_y$ transition is due to B$_A$ and red-most absorbing one to B$_B$ [26,40,45,46,51–53].

The nature of the absorption shoulder at 806 nm observed in the spectrum of Y(M210)W-Pheo RCs at 10 K (Fig. 1) is less clear. The B$_A$ molecule in the residual H$_B$-only exchanged Y(M210)W RCs might have the Q$_y$ transition in this spectral region. However, in view of the high pigment exchange yield ($\leq$ 10% of H$_B$-only exchanged RCs were calculated to be present in the Y(M210)W-Pheo preparations), a contribution of H$_B$-only exchanged RCs is expected to be too small to account for the absorption at 806 nm. Another possibility is that the 806-nm shoulder is dominated by the absorption of the high-energy transition of P [40–42,53–55]. Gray et al. [56] have noted that a 3-nm red shift of the Q$_y$ absorption band of the monomeric BChls (from 803 to 806 nm) observed upon mutation of the tyrosine M210 to phenylalanine or leucine in *Rba. sphaeroides* was accompanied by a 15% increase in the extinction coefficient at 806 nm in the mutant RCs relative to that at 803 nm in wild-type RCs. These authors have suggested that one of the reasons for the observed difference in the extinction coefficient might be more overlap of the Q$_y$ transition of the monomeric BChls and the high-energy exciton component of P in the mutant RCs [56]. Although similar effects seem to be characteristic of the Y(M210)W mutation, the unusually high amplitude of the 806-nm absorption shoulder in Y(M210)W-Pheo RCs makes it difficult to ascribe this feature to the high-energy transition of P, at least without a complicated suggestion on borrowing of dipole strength by the high-energy transition from other transition(s). An alternative and more probable explanation is that Y(M210)W RCs are spectrally heterogeneous and both the 813-and 806-nm bands are due to the BChl$_B$ molecules having different environments of the B$_B$ protein pockets. With this explanation, the 806-nm absorbing B$_B$ molecules would be less accessible to borohydride treatment than that absorbing at 813 nm. The "three-band" structure of the 800-nm feature in Y(M210)W-Pheo RCs is apparently a result of the Y(M210)W mutation and not due to pigment exchange, since this structure was not observed in the 6 K absorption spectrum of pheophytin-modified RCs from *Rba. sphaeroides* R-26 [33].

3.2. Time-resolved absorption difference spectra of Y(M210)W RCs

The effects of the mutation and pigment exchange on charge separation were studied by time-resolved absorption difference spectroscopy in the (sub)picosecond up to nanosecond range at 10 K upon excitation at 900 nm.

For the Y(M210)W RCs (Fig. 3) the overall features in the time-resolved spectra were similar to those reported in the literature [25,26,40,57,58]. The spectra at early time represent primarily the excited state of the primary electron donor P* with distinct signals around 800 nm and a large negative band around 900 nm [5]. The former signals reflect changes in the excitonic interactions between P and the accessory BChls upon formation of P*. The large negative

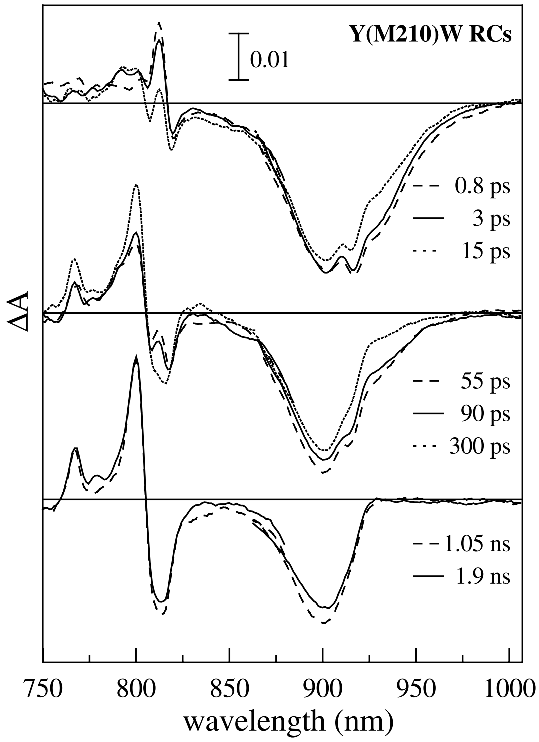

Fig. 3. Time-resolved absorption difference spectra of Y(M210)W RCs at 10 K at various delay times indicated following excitation of P at 900 nm (time resolution 600 fs).

feature can be ascribed to a superposition of the bleaching of the ground-state Q_y absorption band of P and stimulated emission from P*, which is red-shifted with respect to the ground-state absorption band.

Besides the bleaching at about 900 nm, the time-resolved absorption difference spectra at times of up to about 100 ps show distinct minima at the long-wavelength side, at about 917 and 932 nm. Vos et al. [57] have simulated these features by adding to the (bleached) ground-state absorption spectrum an emission spectrum with a variable Stokes shift, shaped as the mirror image of the absorption spectrum. The appearance of the distinct features in the transient spectra was explained by the superposition of the bleaching of a steep ground-state absorption band and a steep stimulated emission band at 10 K [57]. At 77 K the bleaching signal in the P region displayed a smooth band (not shown), which can be explained by the broadening of the spectra at higher temperatures.

At longer delay times (hundreds of picoseconds) the P* stimulated emission signal of the Y(M210)W RCs (Fig. 3) had largely disappeared. The decay of stimulated emission was accompanied by partial recovery of the ground-state absorption of P as indicated by a decrease of the amplitude of the bleaching signal in the region of 880–900 nm, where the contribution of stimulated emission to the difference spectrum is expected to be minimal or absent. The main decay component of stimulated emission had a time constant of about 200 ps, which corresponds to decay of P* by formation of the charge-separated state $P^+H_A^-$ in combination with direct decay to the ground state. A second, much faster decay component of stimulated emission of about 8 ps with relative amplitude of 0.5 was needed to obtain a good fit of the kinetics (not shown). Such a relatively fast component has been observed for almost all RC preparations and was ascribed to a separate relaxation of the excited state causing a shift of stimulated emission to shorter wavelengths [25,58]. Our time constants are in good agreement with those obtained by Nagarajan et al. [25]. Vos et al. [57,58] and van Brederode et al. [40], however, reported a mono-exponential decay of the stimulated emission with a time constant of 320 and about 400 ps, respectively. This difference may be due to differences in the RC preparations: our experiments and those of Nagarajan et al. were performed with detergent-solubilized RCs, whereas Vos et al. and van Brederode et al. used membrane-bound RC complexes. Differences in environment are known to affect the characteristics of electron transfer [58].

The results presented here as well as those reported earlier [25,40,57,58] show that the initial electron-transfer reaction $P^* \rightarrow P^+H_A^-$ in Y(M210)W RCs is very slow, and charge separation occurs at cryogenic temperatures in hundreds of picoseconds. The low rate of charge separation may account for the lack of an observable $P^+H_A^-$ spectrum (Fig. 3), since at 10 K electron transfer forming $P^+Q_A^-$ may be faster than $P^+H_A^-$ formation, so that the population of the latter state would be low. This explanation is consistent with the findings that, in contrast to wild-type RCs for which the initial electron-transfer reaction speeds up at low temperatures, in Y(M210)W RCs the $P^* \rightarrow P^+H_A^-$ reaction slows down with decreasing temperature. Electron transfer from H_A^- to Q_A is not significantly affected by the Y(M210)W mutation and appears to speed up at low temperatures similar to what is observed in wild-type RCs [25].

The final difference spectrum measured in Y(M210)W RCs at a delay of 1.9 ns (Fig. 3) is similar, although not identical, to the spectrum measured at 10 K in the millisecond time-range (Fig. 4, dashed line). Besides the bleaching of the long-wavelength ground-state absorption band of P at about 895 nm, the spectra include absorbance changes due to electrochromic band-shifts of the accessory BChl and BPheo around 800 and 760 nm, respectively. All these features are characteristic for the $P^+Q_A^-$ state formation in both native and mutant RCs [25,33,40,51]. A comparison of the magnitude of the bleaching of the ground-state band of P (at 880–900 nm) at 1.9 ns and shorter times (Fig. 3) indicates that the yield of $P^+Q_A^-$ in Y(M210)W RCs at 10 K is significantly less than 100% (~ 60%). The 60% efficiency of $P^+Q_A^-$ formation (relative to wild-type RCs) has earlier been reported for membrane-bound Y(M210)W RCs at cryogenic temperatures [39].

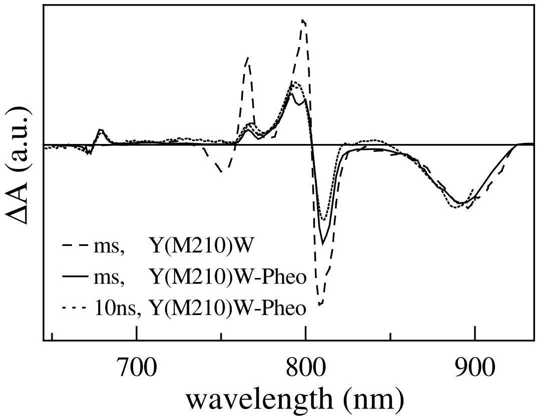

Fig. 4. Absorption difference spectra at 10 K of the $P^{+}Q_{A}^{-}$ state measured in the millisecond time-range in Y(M210)W RCs (dashed line) and Y(M210)W-Pheo RCs (solid line) upon 532-nm excitation. The spectra were obtained by plotting the combined amplitudes of the bi-exponential decay kinetics upon excitation at 532 nm: 6 and 16 ms in Y(M210)W RCs and 10 and 40 ms in Y(M210)W-Pheo RCs. Dotted line: Absorption difference spectrum of Y(M210)W-Pheo RCs at 10 ns after excitation of P (measured with the dye laser setup, the same as for the spectra of Figs. 3 and 5). The spectra were normalized at their P bleaching band.

3.3. Time-resolved absorption difference spectra of Y(M210)W-Pheo RCs

The time-resolved absorption difference spectra obtained with the Pheo-exchanged Y(M210)W RCs at 10 K are shown in Fig. 5. The shape of the early spectra is similar to those of the original Y(M210)W RCs (Fig. 3), with a positive signal at 807 nm, a small negative feature around 815 nm and a bleaching in the P region, indicating the presence of the excited state of P. Compared to the original Y(M210)W RCs, the positions of all peaks were blue-shifted by about 5 nm. Except for changes in amplitude and for small shifts, the shape of the difference spectra did not change significantly up to a delay of about 100 ps, indicating that, like in Y(M210)W RCs, the excited state was very long-lived. At later times the P bleaching decreased, the positive band at 807 nm shifted further to the blue and vanished almost completely, and the negative signal around 815 nm became blue-shifted too. As a result, the difference spectrum measured at 1.9 ns showed mainly two bleaching bands, located at 811 and 890 nm. Stimulated emission from P* showed a bi-exponential decay of about 7 and 490 ps (relative amplitude 0.5:1). At 807 nm the same kinetic components were found. The 7-ps component was mainly associated with a small shift of the bands. The main decay component of almost 500 ps was even slower than in the Y(M210)W RCs. At 1.9 ns there was hardly any stimulated emission left, indicating that the singlet-excited state of P cannot account for the signal at 1.9 ns. Measurements in the microsecond time range did not indicate any significant triplet formation in the Y(M210)W-Pheo RCs (data not shown). Therefore, the difference spectrum measured at 1.9-ns delay must be ascribed to the presence of a charge-separated state. The quantum yield of this state was approximately 30–40%, as indicated by a comparison of the magnitude of the bleaching at 890 nm taken at 1.9 ns and at early delay times (Fig. 5). This rather low quantum yield is obviously a consequence of the very large time constant for the formation of the putative charge-separated state. A time constant of ~ 1.7 ns can be roughly estimated on the basis of the lifetime of P* of about 500 ps, determined from stimulated emission decay, and the yield of 30%. About 60–70% of the excited state population seems to return to the ground state.

The difference spectrum measured at 1.9 ns for the Y(M210)W-Pheo RCs (Fig. 5) is significantly different from the 1.9-ns spectrum obtained under similar experimental conditions for the original Y(M210)W RCs (Fig. 3). Large differences were observed in the region of $B_{A,B}$ absorption. In contrast to the Y(M210)W RCs, the Y(M210)W-Pheo RCs do not show the electrochromic band-shift that is typical for the presence of $P^{+}Q_{A}^{-}$. Only an absorption decrease at 811 nm is observed in the 1.9-ns spectrum of Y(M210)W-Pheo RCs. From these data we conclude that the 1.9-ns absorption difference spectrum in Y(M210)W-Pheo RCs does not represent the $P^{+}Q_{A}^{-}$ state.

Absorption difference spectra measured in Y(M210)W-Pheo RCs at later times after excitation are shown in Fig. 4 by the dotted and solid line. The first spectrum (dotted line) was measured at a delay of 10 ns with the same dye laser pump-probe system as the spectra shown in Fig. 5. The

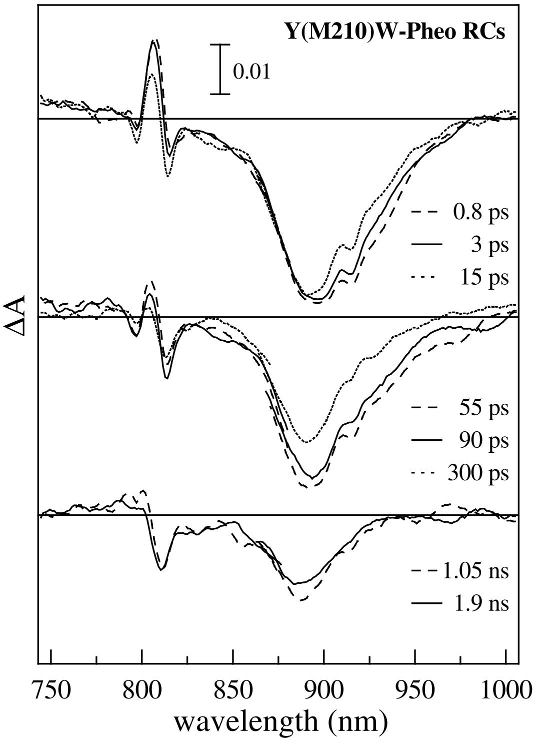

Fig. 5. Time-resolved absorption difference spectra of Y(M210)W-Pheo RCs at 10 K at various delay times indicated following excitation of P at 900 nm (time resolution 600 fs).

spectrum is different from that obtained at 1.9 ns and is in fact almost identical to one measured in the ms range with 532-nm excitation (Fig. 4, solid line). The latter can only be assigned to the state $P^+Q_A^-$ since the Q_B occupancy was less than 5% in the preparations used (see Materials and methods). This means that also the 10-ns spectrum must be due to $P^+Q_A^-$. Both spectra display bleaching of the long-wavelength band of the primary electron donor P at about 895 nm, absorbance changes around 800 nm related to the electrochromic shift of the accessory BChl bands, and absorbance changes due to a red shift of the absorption band of the newly introduced Pheo molecules at about 675 nm. Only a relatively small signal is observed in the Q_y absorption region of the native BPheo molecules around 760 nm. All these spectral features are close to those previously observed in the 10 K absorption difference spectrum for the $P^+Q_A^-$ state in *Rba. sphaeroides* R-26 RCs, in which BPheos at both the H_A and H_B binding sites were exchanged with Pheo [33].

We conclude therefore that the difference spectra in Y(M210)W-Pheo RCs measured at 10 ns and in the millisecond range are dominated by absorbance changes due to formation of $P^+Q_A^-$. Unfortunately, for technical reasons (see Materials and methods), the amplitudes of the three spectra in question cannot be compared with each other (the spectra of Fig. 4 are in fact normalized at 890 nm), and this considerably hinders an interpretation of the origin of the $P^+Q_A^-$ signals. Most probably, there is a contribution from several processes with an overall yield of $P^+Q_A^-$ being small. At least part of the signals may be due to the small fraction (< 10%) of H_B-only exchanged RCs. This fraction of RCs is expected to have electron-transfer properties similar to those of the original Y(M210)W RCs and may contribute already to the 1.9-ns spectrum, since electron transfer to Q_A is completed by that time. However, this contribution to the 1.9-ns signal is calculated to be of minor influence (< 20% of the signal at 890 nm). Another possibility is that the state, causing the dominant contribution to the 1.9-ns spectrum, might disappear between 1.9 and 10 ns partially by charge recombination to the ground state and partially by electron transfer to Q_A to yield $P^+Q_A^-$. We argue below that the 1.9-ns spectrum may be attributed to the $P^+B_B^-$ state formed in the majority (>90%) of RCs containing BPheo both at the H_A and H_B site. In that case, the quantum yield of $P^+Q_A^-$ is expected to be very low due to the long distance between B_B and Q_A. Moreover, a reduction of Q_A via B_B^- is certainly not a naturally designed electron-transfer pathway. In this regard we note that the quantum yield of the $P^+Q_A^-$ state in $H_{A,B}$-exchanged RCs of *Rba. sphaeroides* R-26 formed by direct electron transfer from B_A^- to Q_A was found to be as low as 38% for temperatures below 60 K (the yield of $P^+B_A^-$ was essentially 100%) [9,30]. Finally, although electron transfer in the A-branch of the Y(M210)W RCs with both BPheos exchanged seems to be strongly suppressed at low temperatures due to unfavorable position of the free energy levels of the $P^+B_A^-$ and $P^+\text{Pheo}_A^-$ states (see below), some "leakage" of an electron to Q_A down the A-side cannot be excluded.

3.4. Possible origin of the 1.9-ns absorption difference spectrum in Y(M210)W-Pheo RCs

In order to assign the signal at 1.9 ns in Y(M210)W-Pheo RCs, we have to take into consideration the consequences for the electron-transfer dynamics of (i) the mutation of the tyrosine M210 to a tryptophan and (ii) the exchange of the BPheos (H_A, H_B) with Pheos.

Replacement of the tyrosine M210 by a tryptophan was shown to bring about no gross structural changes [59], but it drastically slows down the initial electron-transfer reaction $P^* \rightarrow P^+H_A^-$, makes this reaction thermally activated [25,26] and reduces the yield of primary charge separation [39,58]. Electron transfer from P* to H_A in the Y(M210)W mutant RCs occurs on the time scale of a few hundreds of picoseconds compared to a few picoseconds in wild-type RCs at cryogenic temperatures. The Y(M210)W mutation was found to increase the midpoint potential of the P/P^+ redox couple by about 50 mV [25,60], which should result in a uniform increase in the free energy of all the charge-separated states on the A- and B-side. This factor contributes to the alterations in primary photochemistry of RCs. However, the changed temperature dependence of the initial electron-transfer reaction can only be explained if the mutation, in addition to destabilization of P^+, also significantly destabilizes B_A^- [25]. A recent crystallographic study [59] has shown that the bulky tryptophan appears to cause a small tilt of the B_A macrocycle, in which a change in geometry may lead to a change in electronic interaction between P and B_A. Such change may contribute to the factors determining the slow rate of primary electron transfer [59]. Theoretical calculations [27] have shown that the effects of the mutation on the free energy of $P^+B_A^-$ appear to be sufficient to raise this state above P*, thereby making the primary electron transfer reaction energetically less favorable. By modeling the results of time-resolved experiments on Y(M210)L mutant RCs of *Rba. sphaeroides*, which exhibits electron-transfer properties similar to those of Y(M210)W RCs, the difference in free energy between P* and $P^+B_A^-$ was determined to be about 60 meV (an energetic distribution of the intermediates of ~ 25 meV was assumed) [61]. In light of this data and considering the low temperature at which our experiments were performed, it is unlikely that the $P^+B_A^-$ state is significantly populated in Y(M210)W-Pheo RCs and, consequently, that the 1.9-ns difference spectrum is associated with this state. This conclusion agrees with the observation that the 1.9-ns spectrum does not show the bleaching at 800 nm that would be expected to be observed if B_A was reduced to B_A^- due to formation of $P^+B_A^-$ [9].

Exchange of BPheo *a* with Pheo *a*, which is more difficult to reduce than BPheo in vitro, significantly raises the free energy level of $P^+H_A^-$ in *Rba. sphaeroides* R-26

RCs, resulting in altered electron-transfer properties of the modified RCs [7,9,28–30]. $P^+Pheo_A^-$ was shown not to be involved in electron transfer in the pheophytin-modified RCs at low temperature, and charge separation was explained by a super-exchange step, directly from $P^+B_A^-$ to $P^+Q_A^-$, with a reduced quantum yield [9,30]. The X-ray crystal structure analysis has demonstrated that pigment exchange affected neither the $B_{A,B}$ nor the P binding site [62]. This fact suggests that the free energy of $P^+B_A^-$ and the rate of the initial electron-transfer reaction ($P^* \rightarrow P^+B_A^-$) are also not significantly altered by pigment exchange. In agreement with the structural data, the results of femtosecond transient absorption studies have shown that the rate of primary charge separation in pheophytin-exchanged RCs was essentially the same as in native RCs, both at room temperature [29] and at 5 K [9]. The free energy level of $P^+Pheo_A^-$ has been estimated [30] to lie by about 25 meV above that of $P^+B_A^-$, which itself is ca. 40–70 meV below P* [7,17,25,29,63–65]. According to Nagarajan et al. [25], the mutation of the tyrosine M210 to a tryptophan raises the free energy of $P^+H_A^-$ by about 70 meV. If the same free energy increase is maintained for $P^+Pheo_A^-$, the combined effects of mutation and pigment exchange seem to be enough to elevate the $P^+Pheo_A^-$ state above P*.

Collectively, the results and estimates presented allow one to conclude that a combination of the Y(M210)W mutation and exchange of BPheo with Pheo raises the free energy levels of $P^+B_A^-$ and $P^+Pheo_A^-$ sufficiently above that of P* to make the A-side of the RC practically incapable of electron transfer at low temperatures.

One suggestion that could explain the origin of the 1.9-ns absorption difference spectrum in Y(M210)W-Pheo RCs is that under conditions when the A-side is shut down the long lifetime of P* results in an increased probability for electron transfer through the B-branch of cofactors. The states $P^+B_B^-$ and/or $P^+Pheo_B^-$ should be invoked in this case. There are, however, several arguments against population of $P^+Pheo_B^-$. (i) It would be impossible to explain the decrease at 811 nm in the 1.9-ns difference spectrum in terms of bleaching of the absorption band of $Pheo_B$ upon formation of $P^+Pheo_B^-$ because the newly introduced plant pheophytin *a* molecules do not absorb in this spectral region (Fig. 1). (ii) An alternative explanation that the 811-nm absorption decrease reflects a re-distribution of the dipole strengths between the pigments due to disruption of exciton interactions caused by $Pheo_B$ reduction is unlikely as well. Indeed, the difference in site energy of the Q_y transition of $Pheo_B$ and that of B_B is too large (675 versus 806–813 nm) to expect considerable resonance interactions between these transitions. (iii) $P^+Pheo_B^-$ formation is expected to cause an electrochromic shift of the B absorption band, based on analogy with the $P^+BPheo_A^-$ radical pair (see for example Ref. [40]). As we have already mentioned this is clearly not the case.

We propose that the formation of $P^+B_B^-$ is the more likely explanation for the origin of the 1.9-ns spectrum. It is supported by two spectroscopic observations. First, like $P^+B_A^-$ registered at 5 K in Pheo-modified RCs of *Rba. sphaeroides* R-26 [9], the state responsible for the 1.9-ns difference spectrum of Y(M210)W-Pheo RCs does not exhibit the electrochromic shift of the B absorption band that normally is associated with P^+ formation. Also, placing a bacteriopheophytin molecule (ϕ_B) instead of B_B in the H(M182)L mutant resulted in formation of the $P^+\phi_B^-$ state, which appeared to lack the electrochromic shift of the B band [18]. The close proximity of P^+ and ϕ_B^- was suggested to decrease the electric field strength felt by B_A, thereby decreasing the magnitude of its electrochromic shift [18]. Though the reasons for the lack of the electrochromic band-shift of the accessory bacteriochlorophyll molecule not involved in electron transfer (B_B in Pheo-modified *Rba. sphaeroides* R-26 RCs and B_A in H(M182)L mutant RCs) need further investigations, the similarity in the difference spectra points out that the state formed at 1.9 ns has the same charge-transfer character as $P^+B_A^-$ and $P^+\phi_B^-$. Second, the absorption decrease at 811 nm corresponds to bleaching of the ground-state band on the red-most side of the $B_{A,B}$ absorption, where B_B has the Q_y transition at 806–813 nm. Based on the above considerations we attribute, tentatively, the 1.9-ns absorption difference spectrum in Y(M210)W-Pheo RCs to the $P^+B_B^-$ state, with the bleaching signal around 890 nm due to photo-oxidation of P and that at 811 nm to reduction of B_B. Plausibly, the bleaching signal at 811 nm is a composite of the bleachings of the absorption bands at 806 and 813 nm, whose bands seem to belong to the B_B molecules having different environments of the B_B protein pockets.

A lifetime of several nanoseconds can be roughly estimated for the state giving rise to the 1.9-ns spectrum, based on its disappearance between 2 and 10 ns. This value appears to be slightly longer than the time constant on the order of 1 ns expected for charge recombination of $P^+B_B^-$, based on analogy with $P^+B_A^-$ [9,19], and it is at least 10 times larger than the lifetime of about 200 ps reported for the $P^+\phi_B^-$ state in quinone removed or reduced H(M182)L mutant RCs of *Rba. sphaeroides* [18]. This difference in lifetimes, however, does not rule out that the state responsible for the 1.9-ns spectrum is $P^+B_B^-$. Indeed, to our knowledge there is no experimental data on a lifetime of the $P^+B_B^-$ state in the native RC. The H(M182)L mutant contains a bacteriopheophytin molecule (ϕ_B) instead of the natural B_B, and it is unclear at present to what extent pigment replacement affect the lifetime of the associated charge-separated state. Note also that, as we discuss below, the Y(M210)W mutation seems to alter the protein environment of the B_B molecule, whose alteration might have an effect on the $P^+B_B^-$ lifetime in our preparations.

Theoretical calculations of radical pair energies based on the crystal structure of *Rhodopseudomonas viridis* RCs have placed $P^+B_A^-$ isoenergetic, below [27,66] or above [67] P*, but consistently resulted in an estimate of the free energy level of $P^+B_B^-$ lying significantly above P* [66,67] (about 240 meV, according to Parson et al. [66]). The phenolic OH dipole of tyrosine M210 was found to considerably stabilize B_A^-, whereas the symmetrically positioned phenylalanine L181 does not provide a similar stabilization of B_B^- [27]. The high free energy of $P^+B_B^-$ relative to P* and $P^+B_A^-$ is thought to be an important factor responsible for the functional inactivity of the B-branch of cofactors [66,67]. Recent observations of B-side electron transfer in H(M182)L mutant RCs [18] and in wild-type *Rba. sphaeroides* RCs at high excitation intensity [23] appear to support the idea that in these types of RCs $P^+B_B^-$ is indeed higher in energy than P*. From room temperature measurements of the long-lived fluorescence decay in H(M182)L mutant RCs, the standard free energy of the $P^+\phi_B^-$ state has been estimated that allowed to estimate the standard free energy of the $P^+B_B^-$ state in wild-type RCs to be 0.1 ± 0.07 eV above P* [68]. Based on this data one would expect that the free energy level of $P^+B_B^-$ in Y(M210)W RCs is significantly above that of P* as well. The results we presented here are clearly not consistent with this scenario. An attribution of the 1.9-ns state in Y(M210)W-Pheo RCs to $P^+B_B^-$, which forms with a yield of 30–40% at 10 K and lives several nanoseconds, suggests that in this mutant $P^+B_B^-$ is located close or below P*. Another possibility is that the RCs are heterogeneous and that there exists a manifold of $P^+B_B^-$ states extending both above and below P* in energy. It seems reasonable to expect that replacement of the native $BPheo_B$ by Pheo raises $P^+Pheo_B^-$ in energy but does not significantly influence the free energy of $P^+B_B^-$. We infer therefore that the proposed lowering of the free energy level of $P^+B_B^-$ in Y(M210)W-Pheo RCs relative to that in native RCs is a result of the Y(M210)W mutation. The mutation at this position might have a specific long-distance effect on the RC protein, resulting in changes of the environment of the B_B binding pocket and, as a consequence, in alteration of the spectral and redox properties of B_B. Our data do not provide a direct evidence for a change of the reduction potential of B_B. However, the complicated structure of the 10 K ground-state absorption spectrum of Y(M210)W-Pheo RCs in the $B_{A,B}$ region (Fig. 1), which to our knowledge has not been reported for native RCs or other types of mutant RCs, is consistent with a significant perturbation of the environment of the B_B protein pocket upon mutation. Although a perturbation of the environment decreases the free energy level of $P^+B_B^-$, allowing its population at 10 K, the rate of electron transfer from P* to B_B is very low as it is indicated by the very large time constant for formation of the 1.9-ns state in Y(M210)W-Pheo RCs (ca. 1.7 ns). Slow formation of $P^+B_B^-$ in Y(M210)W-Pheo RCs within 1.7 ns might be due to slow relaxation of surrounding molecules leading to lowering the $P^+B_B^-$ energy level and stabilization of the charge-separated state. This is in contrast to fast (~ 1.5 ps at 10 K) relaxation of surrounding molecules in the A-branch of wild-type RCs.

It is worth noting that the abovementioned possibility of a long-range effect of mutations on spectral and electron-transfer properties of bacterial RCs is not unprecedented. Recently DiMagno et al. [69] have found the effects of distant mutations on the absorption spectra of the chromophores and the rate of primary electron-transfer in RCs from the quadruple mutant strain RQ of *Rba. capsulatus*. In this particular strain, mutations of polarizable amino acid residues near the quinone binding sites were shown to lead to spectral shifts of the Q_y and Q_x absorption bands of the RC bacteriochlorophylls, and result in reduction of the rate of the $P^* \rightarrow P^+H_A^-$ reaction. The distinct effects were observed despite the introduced quinone-site mutations are ~ 35 Å distant from the P or B bacteriochlorophylls, and structural changes, if any, are minor and are localized to the Q_A-binding pocket [69].

4. Conclusions

We have shown that mutation of tyrosine M210 to tryptophan, combined with replacement of bacteriopheophytins H_A and H_B by plant pheophytin, brings about a practically complete stop of electron transfer in the A-branch of *Rba. sphaeroides* RCs at 10 K. The lifetime of P* is increased to 500 ps, and from this long-lived excited state a new charge-separated state is formed with a yield of about 30%. The most probable candidate for this charge-separated state is, as it follows from spectroscopic observations, $P^+B_B^-$. Analysis of the data on the basis of current knowledge of the spectroscopy and dynamics of bacterial RCs allows one to infer that the free energy level of $P^+B_B^-$ in Y(M210)W-Pheo RCs is lowered relative to that in native RCs due to alteration of the protein environment of the B_B molecule, caused by the Y(M210)W mutation. A significant perturbation of the environment of the B_B binding pocket upon mutation is also suggested by the unusual structure of the 10 K ground-state absorption spectrum of Y(M210)W-Pheo RCs in the region of the Q_y absorption of the monomeric bacteriochlorophylls. We conclude therefore that replacement of the tyrosine M210 by a bulky tryptophan can significantly affect the absorption and redox characteristics of the B_B bacteriochlorophyll due to a long-range effect on the RC protein. It is still unclear whether and to what extent similar effects are observed in other types of amino acid substitutions at the M210 position. However, a principal possibility of such effects should be taken into account in explaining spectral and electron-transfer properties of this very interesting family of mutants that contain modifications of one of the key residues in bacterial RCs.

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