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Primary electron transfer kinetics in bacterial reaction centers with modified bacteriochlorophylls at the monomeric sites $B_{A,B}$

(photosynthesis/femtosecond spectroscopy/*Rhodobacter sphaeroides*)

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ABSTRACT **The primary electron transfer has been investigated by femtosecond time-resolved absorption spectroscopy in two chemically modified reaction centers (RC) of *Rhodobacter sphaeroides*, in which the monomeric bacteriochlorophylls B_A and B_B have both been exchanged by 13^2-hydroxybacteriochlorophyll a or [3-vinyl]-13^2-hydroxybacteriochlorophyll a. The kinetics of the primary electron transfer are not influenced by the 13^2-hydroxy modification. In RCs containing [3-vinyl]-13^2-hydroxybacteriochlorophyll a the primary rate is reduced by a factor of 10.**

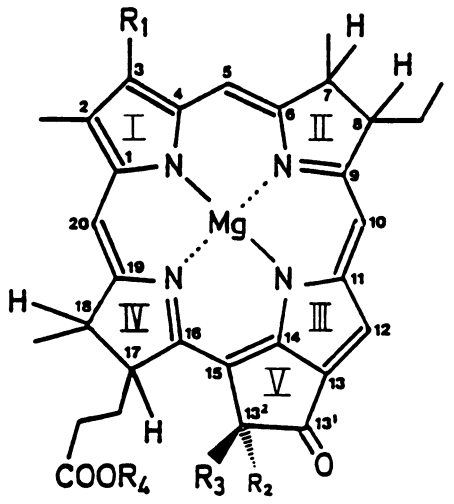
The three known x-ray structures of bacterial reaction centers (RCs) show basically the same arrangement of the cofactors (see refs. 1–4). They form two branches [A (active) and B (inactive)], which are arranged in an approximate twofold symmetry. Surprisingly, the electron transfer (ET) has been found to proceed exclusively along the A branch (5–7). From the primary donor, P870 or P960, an electron is transferred to the acceptor quinone (Q_A). The structural arrangement of the chromophores suggests an ET via the two intervening pigments—i.e., the monomeric bacteriochlorophyll (BChl) B_A and the bacteriopheophytin (BPhe) H_A. While there is general agreement that the BPhe H_A is an intermediate electron acceptor (5–9), the role of the BChl B_A located between the BChl dimer P and the BPhe H_A is still controversially discussed. First studies with sufficient time resolution and an appropriate excitation wavelength led to the conclusion that the state $P^+B_A^-$ (P, primary donor) is not a real intermediate in the ET (5, 10, 11). However, a direct ET from P* to H_A over an edge-to-edge distance of ≈10 Å can be accounted for only by a "superexchange" transfer via a virtual intermediate $P^+B_A^-$ (12–15).

The observation of an additional, subpicosecond time constant in *Rhodobacter sphaeroides* (16–20) again raised the possibility of $P^+B_A^-$ being a real intermediate according to $P^* \xrightarrow[3.5\ ps]{} P^+B_A^- \xrightarrow[0.9\ ps]{} P^+H_A^- \xrightarrow[200\ ps]{} P^+Q_A^-$. This model is suggested by the structural arrangement of the pigments and is supported by time-resolved linear dichroism data (17). However, other models cannot be excluded at the moment: the additional kinetic component could also be due to a relaxation of a primary excited electronic state P* (17, 18) or a microheterogeneity of the RCs (21).

In this paper, we present kinetic absorption studies on RCs containing 13^2-hydroxy-BChl-a or [3-vinyl]-13^2-hydroxy-BChl-a at the sites $B_{A,B}$ of the monomeric BChls (for structures see below) giving additional information on the primary ET.

MATERIAL AND EXPERIMENTAL TECHNIQUES

RCs from *Rb. sphaeroides* R26.1 were prepared according to standard procedures (22); RCs from the wild-type strain ATCC 17023 were prepared as described (16). RCs containing 13^2-hydroxy-BChl-a and [3-vinyl]-13^2-hydroxy-BChl-a were prepared according to Struck *et al.* (22, 23). Q molecules lost during the purification procedure were replaced. The BChl-a exchange yielded values of 50% ± 5% for 13^2-hydroxy-BChl-a. Since the two BChl-a molecules of the primary donor P do not exchange (24), this corresponds to a complete exchange of the monomeric pigments at sites B_A and B_B. The respective value for [3-vinyl]-13^2-hydroxy-BChl-a is 40% ± 5% corresponding to an average exchange of 80% at sites B_A and B_B (see *Discussion*).



R_2= $COOCH_3$ R_4= $C_{20}H_{39}$

	R_1	R_3
BChl a	$COCH_3$	H
13^2-OH-BChl a	$COCH_3$	OH
[3-vinyl]-13^2-OH-BChl a	$CHCH_2$	OH

Kinetic measurements were performed at $T \cong 298$ K in cuvettes of 1-mm path length under stirring. The sample volume was 0.2–0.3 ml. The transmission was between $T \cong$ 10% and 50% at λ = 860 nm (80–25 μM). The experimental

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Abbreviations: RC, reaction center; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; ET, electron transfer; P^+, B_A^-, and H_A^-, anion and cation radicals of primary donor P, monomeric BChl B_A, and BPhe H_A, respectively; Q, quinone; subscripts A and B, active and inactive branches.

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system has been described in detail (19). Characteristics of the excitation pulses: λ = 860 or 875 nm ($\Delta\lambda$ = 20 nm); energy, 1 μJ; duration, 200 fs; spot size, 1 mm (chosen to excite not more than 15% of the RCs in the irradiated volume by each laser pulse). Probing pulses: 20-nm-wide portion of a femtosecond white light continuum ($t_{probe} \cong 200$ fs), parallel polarizations of exciting and probing pulses. The signal points were modeled by a sum of exponentials convoluted with the experimental response function (for details see ref. 17).

RESULTS

RCs Containing 13^2-Hydroxy-BChl-a at Binding Sites $B_{A,B}$. The absorption spectrum of the 13^2-hydroxy-BChl-a-containing RCs (13^2-OH-RCs) is similar to that of native RCs. A small shift is found in the Q_x absorption band of the monomeric BChls (22). The transient absorption changes were recorded at three significant probing wavelengths λ_{pr}, which well characterize the different kinetic components of the transient absorption changes in native RCs (16). In Fig. 1, the normalized transient absorption changes for 13^2-OH-RCs (solid circles) and for native wild-type RCs (open circles) are plotted for λ_{pr} = 920 nm, λ_{pr} = 785 nm, and λ_{pr} = 665 nm. The kinetic traces at all three probing wavelengths are found to be indistinguishable from the wild-type data within our signal/noise ratio. Accordingly, they can well be accounted for by using the three time constants of 3.5, 0.9, and 200 ps known from investigations on wild-type RCs (16).

RCs Containing [3-Vinyl]-13^2-Hydroxy-BChl-a at Binding Sites $B_{A,B}$. The preparation of RCs containing the modified [3-vinyl]-13^2-hydroxy-BChl-a shows a substantially different absorption spectrum from native RC (Fig. 2*b Inset*). The absorption bands of the modified (monomeric) BChls $B_{A,B}$ are shifted from 802 to 772 nm [Q_y(B) band] and from 600 to 573 nm [Q_x(B) band]. However, around 801 nm, at the peak position of the Q_y(B) band in native RCs, there is a shoulder in the shifted Q_y(B) band of the [3-vinyl]RC preparation.** This shoulder is due to an incomplete exchange (80%) of the monomeric BChl-a. Part of this shoulder may also be due to the upper excitonic component of the Q_y(P) band (22, 25, 26).

Time-resolved measurements were performed at eight different probing wavelengths. The decay of the electronically excited state P* in the gain region at λ_{pr} = 920 nm (Fig. 2*a*) cannot be fit monoexponentially (Fig. 1*a*) with a 3.5-ps time constant. The best monoexponential fit is found for $\tau_1 \cong 17$ ps (Fig. 2*a*, dashed line). However, only a biexponential model function is able to trace the data reasonably well. When we use τ_1 = 3.5 ps taking into account a fraction of unaltered RCs, we obtain the best simulation for a second time constant of $\tau_1' \cong 32$ ps (Fig. 2*a*, solid line) with an amplitude ratio of a_1 (3.5 ps)/a_1' (32 ps) of $\approx$1:2.

To ascertain that the 32-ps kinetic component does not describe a simple relaxation of P* back to the ground state P, we measured the transient absorption change at λ_{pr} = 850 nm (Fig. 2*b*), where the light-induced absorption decrease is mainly due to the bleaching of the Q_y(P) absorption. Only a small amount of ΔA ($\leq$20%) recovers with τ_1 = 32 ps and may be due to stimulated emission from the excited state P*. The remaining absorption decrease is related to the disappearance of P absorption upon formation of the cation radical P^+. The high degree of bleaching at late delay times (t_D = 1 ns) excludes the possibility that the 32-ps component is related to internal conversion. We conclude therefore that the slower component (τ_1' = 32 ps) in the [3-vinyl]RCs reflects a real ET step.

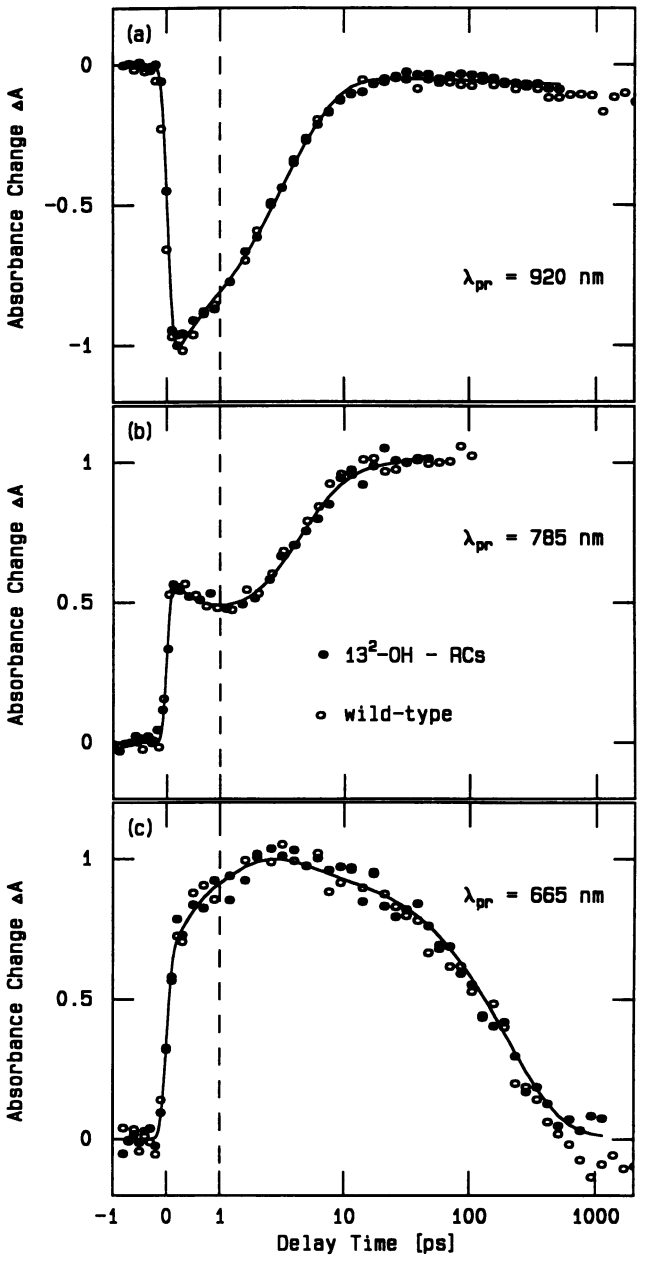

FIG. 1. Transient absorption data (λ_{exc} = 860 nm) on 13^2-OH-RCs (●) and wild-type RCs (○) for probing wavelengths of 920 nm (*a*), 785 nm (*b*), and 665 nm (*c*). Curves are calculated with a sum of exponentials using time constants of τ_1 = 3.5 ps, τ_2 = 0.9 ps, τ_3 = 200 ps, and $\tau_4 = \infty$. Delay time scale is linear for $t_D < 1$ ps and logarithmic for $t_D > 1$ ps.

Further probing wavelengths were chosen to cover the Q_y absorption from native and [3-vinyl]RCs (λ_{pr} = 801, 785, and 770 nm; see Fig. 3). The transient changes of absorption in this region are completely different from those obtained on native RCs (e.g., compare Fig. 1*b* and Fig. 3*b*). For none of these three wavelengths is it possible to simulate the data solely with the three time constants known from native RCs. Introduction of the time constant of τ_1' = 32 ps determined in the measurement at λ_{pr} = 920 nm gives a satisfactory simulation of the data. However, the amplitudes obtained in this way are not consistent with a considerable fraction of unexchanged RCs. One could overcome this problem by introducing an additional component with a time constant of

**The expression [3-vinyl]RC preparation is used for the whole heterogeneous sample (an amount of $\leq$40% of the RCs in the investigated samples may contain unaltered BChl-a at site B_A). The fraction containing [3-vinyl]-13^2-OH-BChl-a at site B_A is called [3-vinyl]RCs.

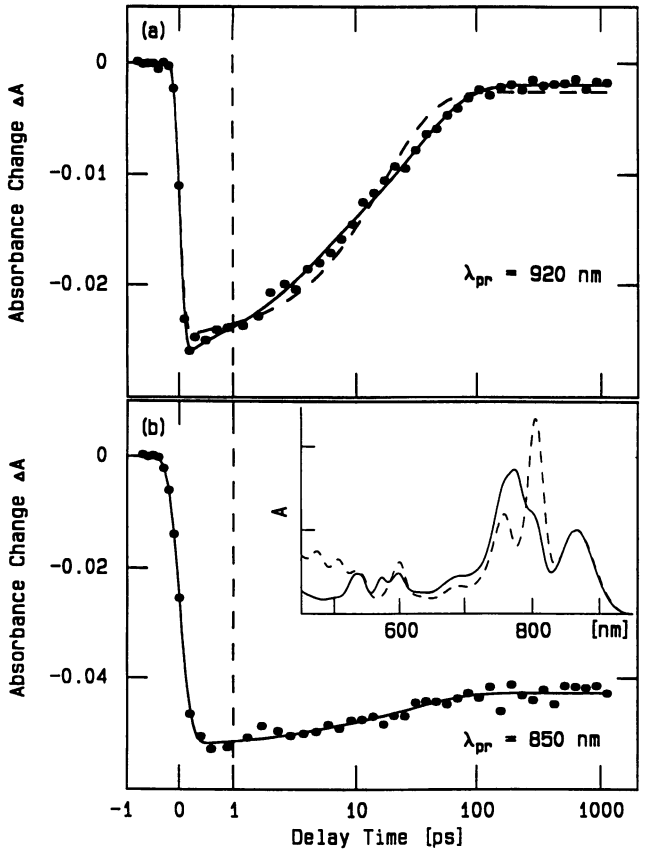

FIG. 2. Transient absorption data (λ_{exc} = 860 nm) on the [3-vinyl]-13²-OH-RC preparation (●). (*a*) Probing wavelength, 920 nm; ---, best monoexponential fit (time constant τ_1 = 17 ps); ——, biexponential model function using time constants of τ_1 = 3.5 ps and τ_1' = 32 ps (amplitude ratio $a_1/a_1' \cong 1{:}2$). (*b*) Probing wavelength of 850 nm. (*Inset*) Absorption spectra of the [3-vinyl]-13²-OH-RC preparation (——) and ATCC 17023 wild-type RCs (- - -). Spectra are normalized to A_{860} = 1. Differences at $\lambda < 540$ nm are due to absorption of a carotenoid present in the ATCC 17023 RCs.

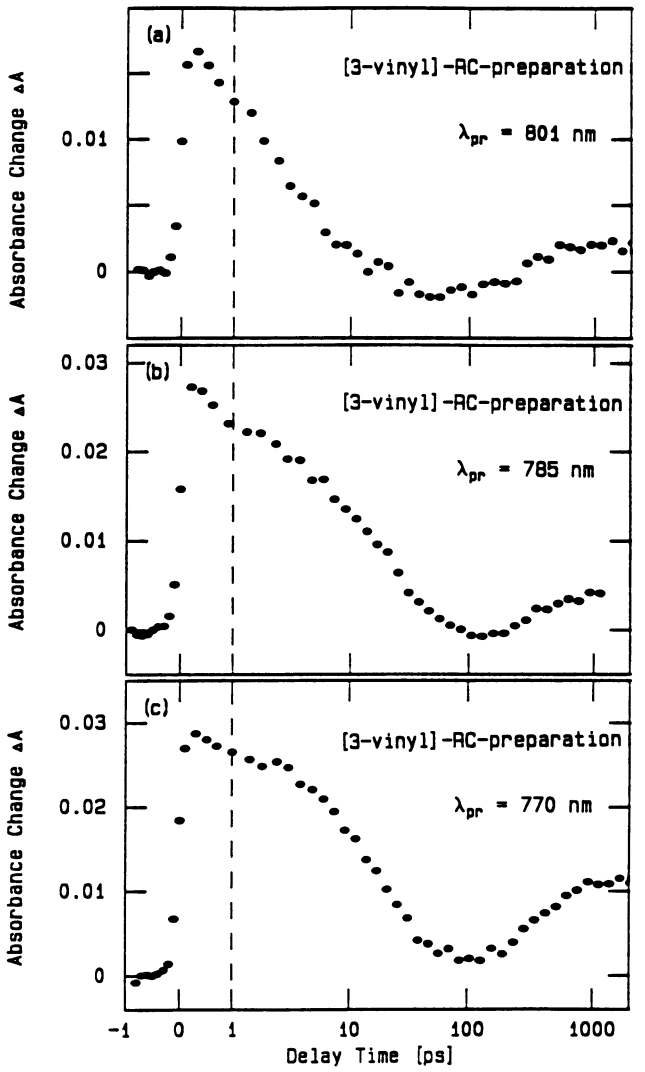

FIG. 3. Transient absorption data (λ_{exc} = 860 nm) on the [3-vinyl]-13²-OH-RC preparation (●) for probing wavelengths of 801 nm (*a*), 785 nm (*b*), and 770 nm (*c*).

several picoseconds in the [3-vinyl]RCs. However, the amount and accuracy of the experimental data do not justify data modeling with five time constants and five corresponding amplitudes.

Measurements at probing wavelengths of λ_{pr} = 665, 655, and 645 nm (data not shown) gave three noteworthy results. (*i*) A small amount of a fast kinetic component ($\tau_2 \cong 1$ ps) is present in the [3-vinyl]RC preparation. (*ii*) The kinetic component with $\tau_1' \cong 32$ ps also appears in this spectral region. (*iii*) At all three wavelengths, there is a considerable contribution of a kinetic component with a time constant of $\tau_3 \cong 200$ ps.

DISCUSSION

RCs Containing 13²-hydroxy-BChl-a. 13²-OH-BChl-a differs from BChl-a in several important aspects. (*i*) It contains an additional hydrophilic substituent at ring V, which can act as both hydrogen bond donor and acceptor. Inspection of the crystal structures has indicated that in the native configuration [13²(*S*) ≡ OH] this substituent can be accommodated without significant distortions. At site B_A the OH group of Ser-173 is positioned within hydrogen-bonding distance and at site B_B the peptide carbonyl group of Gly-200 may act as a partner. (*ii*) There exist two stereoisomers [13²(*S*/*R*)], which in contrast to those of BChl-a are not readily interconvertible. The 13²(*S*) epimer is formed in a 5:1 excess. It also appears to be preferentially exchanged into sites $B_{A,B}$.

The kinetic data show that the substituent change allows efficient ET and leaves all kinetics unchanged up to the state $P^+Q_A^-$. Obviously, there are no critical rearrangements of the protein induced by the chromophor exchange. (*iii*) Replacement of the 13²-hydrogen by a hydroxyl group inhibits enolization of the β-ketoester system present in all (B)Chls and (B)Phes in RCs. There have been numerous investigations on the chemistry of this group (27–29) and its potential functional importance in photosynthesis (30–33). The ready replacement of 13²-hydroxy-BChl-a into sites B_A, B_B and the steady-state spectroscopic similarities between 13²-hydroxy-BChl-a $B_{A,B}$ RCs and native ones were already strong indications that enolization of the pigments at these sites is not important in the ground state, in the charge separated state $P^+Q_A^-$ (H. Käss, J. Rautter, W. Zweygurt, A.S., H.S., and W. Lubitz, unpublished data), and in the triplet state (J. Greis, A.S., H.S., and A. Angerhofer, unpublished data). The kinetic results shown here give very strong evidence that enolization is not even transiently involved in the charge separation process. Within the limits of error, all kinetic properties at times up to 1 ns are indistinguishable from native RCs and can be fitted by the same set of kinetic constants (τ_1 = 3.5 ps, $\tau_2 \cong 0.9$ ps, and τ_Q = 200 ps) and amplitudes. Irrespective of the kinetic model, the replacement of 13²-H by OH is of no consequence to the ET process.

Since 13^2-hydroxylation often has preparative advantages, the second pigment investigated in detail was the doubly modified [3-vinyl]-13^2-hydroxy-BChl-a.

RCs Containing [3-Vinyl]-13^2-Hydroxy-BChl-a. The transient data on the [3-vinyl]RC preparation indicate that it is heterogeneous: ≅65% of the RCs have [3-vinyl]BChl-a at the monomeric site B_A while ≅35% of the RCs have BChl-a at this position.†† The fraction containing [3-vinyl]BChl-a at site B_A exhibits a slower decay of the excited electronic level P* ($\tau_1' \cong 32$ ps). The additional observation of a long-lasting bleaching of the Q_y(P) absorption band gives evidence that an oxidized special pair P^+ is formed. In other words, in the [3-vinyl]RCs the reaction speed of the first ET step is almost 10 times slower than in native RCs. From the experimental data it cannot be decided unambiguously whether or not an additional kinetic constant corresponding to the 0.9-ps component in native RCs is present. The ET reaction from the BPhe H_A to the quinone Q_A is unaffected since the corresponding kinetic has the same 200-ps time constant as in native RCs.

According to our present knowledge the ET in native RCs is in principle compatible with two different reaction schemes (17, 18):

Scheme A. The first reaction is the decay of the electronically excited state P* with 3.5 ps building up the radical pair $P^+B_A^-$, which then decays with a time constant of ≅0.9 ps to $P^+H_A^-$. From there, the electron is transferred to the quinone Q_A with a time constant of 200 ps. Because the second step is faster than the first, the intermediate $P^+B_A^-$ is only weakly populated and therefore difficult to detect.

Scheme B. The fast kinetic is related to an S_1 relaxation process of P*. After this fast relaxation, the real ET starts with the 3.5-ps kinetic bringing the electron directly to the BPhe H_A followed by ET to Q_A. In this scheme, the high speed of the long-distance ($d \cong 10$ Å) transfer from P* to H_A can be explained only if the monomeric BChl B_A mediates the ET as a virtual electron carrier in a superexchange process.

The data on the [3-vinyl]RC preparation give further information on the applicability of these two reaction schemes. The 0.9-ps kinetic detected in native RCs shows up only very weakly in the 650-nm regime in the measurements on the [3-vinyl]RC preparation. Most likely, the weak 0.9-ps component detected around 650 nm is due to the amount of ≈30% of native RCs. The difficulties in resolving the fast component in the [3-vinyl]RC preparation give additional arguments in favor of reaction Scheme **A**. In Scheme **B**, the fast kinetic component would be related to an S_1 relaxation of the excited special pair P*. Since the absorption spectrum, linear and circular dichroism experiments (H. Käss, J. Rautter, W. Zweygurt, A.S., H.S., and W. Lubitz, unpublished data; J. Breton, A.S., and H.S., unpublished data), and ENDOR (J. Greis, A.S., H.S., and A. Angerhofer, unpublished data) experiments give no evidence for any change in the energetics and/or structure of P in the [3-vinyl]RCs, the hypothetical S_1 relaxation process in state P* of Scheme **B** should not be affected by the exchange of the monomeric BChls—i.e., time constant and amplitude of the 0.9-ps component should be unchanged. According to Scheme **B**, in which the 0.9-ps component precedes the 32-ps component, the fast kinetic should show up clearly in the [3-vinyl]RC preparation. Thus, the apparent absence of a 0.9-ps kinetic component in the [3-vinyl]RC gives an indication that an S_1 relaxation process is not responsible for the fast kinetic component. As a consequence, this finding supports the idea that the ET mechanism in the native RCs is a sequential one according to reaction Scheme **A**.

††This amplitude ratio deviates slightly from the average exchange rate mentioned above, indicating that the exchange yields of the BChl-a molecules are different on the two pigment branches.

Despite the arguments and findings described above, we are not able to decide whether the radical pair $P^+B_A^-$ is a real intermediate in the [3-vinyl]RCs, because we cannot rule out or prove the presence of a fast kinetic component. Within our present experimental accuracy, an intermediate $P^+B_A^-$ in the [3-vinyl]RCs is not detectable if its lifetime is shorter than ≈8 ps (assuming a P* lifetime of 32 ps). Thus, the experimental results are compatible with two different ET models for the [3-vinyl]RCs:

$$P^* \xrightarrow{32\ ps} P^+B_A^- \xrightarrow{<8\ ps} P^+H_A^- \xrightarrow{200\ ps} P^+Q_A^-$$

Model I

$$P^* \xrightarrow{32\ ps} P^+H_A^- \xrightarrow{200\ ps} P^+Q_A^-$$

Model II

In both models the influence of the exchange of B_A on the ET from the donor P to the acceptor H_A could be based on two mechanisms (12–14): (*i*) The exchange of the BChl may lead to secondary structural changes in the RCs, which could reduce the reaction speed. (*ii*) The new kind of BChl may have a different electronic wavefunction, which changes the electronic coupling with the neighboring chromophores. It may also lead to different energetics of the radical pair $P^+B_A^-$, which changes the Franck–Condon factor for the ET.

There are several experimental observations arguing against structural changes. The experimental results for 13^2-OH-BChl-a-containing RCs demonstrate that neither the exchange procedure itself nor the 13^2-OH substituent influences the primary kinetics. As the reaction speed is probably very sensitive to structural changes, the latter are unlikely. If the vinyl group itself leads to structural changes, they should show up not only in reaction dynamics but also in the absorption, circular dichroism, or ENDOR spectra of P. All observations seem to exclude structural changes of P: (*i*) The Q_y(P) absorption band of the special pair P is unchanged between native RCs and [3-vinyl]RCs. (*ii*) Circular dichroism (23) and ENDOR experiments (unpublished data) show no significant changes at $\lambda > 840$ nm. There is also some evidence for the absence of a structural change at the binding sites from the fact that Bchl-a and [3-vinyl]Bchl-a show the same wavelength shift upon transferring the pigments from diethylether into RCs (23). As a consequence, we may exclude—at first approximation—structural changes as a cause of the deceleration of the primary ET.

An important difference is that [3-vinyl]Bchl-a should have a higher negative redox potential, by which the free energy of state $P^+B_A^-$ should be increased (replacement of the electron-withdrawing 3-acetyl group). The consequences of the raised energy P^+B^- are as follows:

(*i*) In a two-step model (Model I), the energy of the intermediate state $P^+B_A^-$ of the [3-vinyl]RCs may be close to or even above that of P*. This reduces the Franck–Condon factor. The ET rate would slow down.

(*ii*) In the superexchange model (Model II), the energy of state $P^+B_A^-$ is supposed to lie above that of state P*. An increase of the energy difference between P* and $P^+B_A^-$ reduces the transfer rate.

Both models are compatible with the results in native RCs. The molecular interpretation given above is related to the one used to explain the slowing down of the primary ET rate in genetically mutated RCs where the polar tyrosine M210 was replaced by nonpolar amino acids. Here the slowing down of the reaction speed was also attributed to the changed energetics (34–37). The somewhat weaker slowing down of the decay of P* in mutated RCs (compared to the [3-vinyl]RCs) suggests that the [3-vinyl] modification yields a stronger increase in the energy of the intermediate $P^+B_A^-$.

In conclusion, transient absorption spectroscopy of the primary ET reaction of modified RCs yielded (*i*) RCs where the monomeric BChls are exchanged by 13^2-OH-Bchl-a or [3-vinyl]-13^2-OH-Bchl-a are functionally active. (*ii*) A hydroxyl group at position 13^2 in ring V instead of a proton does not influence the primary ET kinetics. (*iii*) The speed of the primary ET reaction is strongly reduced when a vinyl group replaces the acetyl group at position 3 of ring I, presumably because of an increase in the free energy of the radical pair state $P^+B_A^-$. (*iv*) The lack of detecting a fast kinetic component ($\cong$0.9 ps) in the [3-vinyl]-13^2-OH-RCs argues against an ET scheme for the native RCs in which the fast kinetic component is assigned to a vibrational relaxation in the electronically excited state P*.

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