Early Bacteriopheophytin Reduction in Charge Separation in Reaction Centers of *Rhodobacter sphaeroides*

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ABSTRACT A question at the forefront of biophysical sciences is, to what extent do quantum effects and protein conformational changes play a role in processes such as biological sensing and energy conversion? At the heart of photosynthetic energy transduction lie processes involving ultrafast energy and electron transfers among a small number of tetrapyrrole pigments embedded in the interior of a protein. In the purple bacterial reaction center (RC), a highly efficient ultrafast charge separation takes place between a pair of bacteriochlorophylls: an accessory bacteriochlorophyll (B) and bacteriopheophytin (H). In this work, we applied ultrafast spectroscopy in the visible and near-infrared spectral region to *Rhodobacter sphaeroides* RCs to accurately track the timing of the electron on B_A and H_A via the appearance of the B_A and H_A anion bands. We observed an unexpectedly early rise of the H_A^- band that challenges the accepted simple picture of stepwise electron transfer with 3 ps and 1 ps time constants. The implications for the mechanism of initial charge separation in bacterial RCs are discussed in terms of a possible adiabatic electron transfer step between B_A and H_A , and the effect of protein conformation on the electron transfer rate.

INTRODUCTION

During ultrafast photochemical charge separation in the Rhodobacter (Rba.) sphaeroides reaction center (RC), the primary donor of electrons is the first singlet excited state of a pair of excitonically coupled bacteriochlorophyll a (BChl), termed P*. The first clearly resolved electron acceptor is a bacteriopheophytin a (BPhe, a demetallated bacteriochlorin) termed H_A (1,2). An intervening monomeric BChl, BA, makes direct atomic contact with both P and H_A (shortest center-center distances between atoms in adjacent macrocycles on the order of 3.7-3.8 Å; Fig. 1 *a*). It is generally accepted that P* decays with a lifetime of \approx 3 ps to form the radical pair P⁺B_A⁻, followed by a more rapid electron transfer to H_A in ≈ 1 ps to form $P^+H_A^-$ (3). A third electron transfer to a ubiquinone (Q_A) with a lifetime of ≈ 200 ps produces a membrane-spanning radical pair, $P^+Q_A^-$, that is stable on a millisecond timescale (Fig. 1 b).

Although there is general agreement regarding the timescale of this electron transfer process, the precise mechanism of charge separation is still under debate. In particular, there is uncertainty regarding the possible role of quantum coherence and the extent to which protein conformational changes may affect ultrafast electron transfer. Ultrafast photosynthetic energy transfer and charge separation take place on a timescale in which quantum effects could exert an influence, and the relevance of quantum coherence for biological energy transfer in a variety of light-harvesting complexes has been highlighted (4–11). Harvested light energy is trapped by the charge separation

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that takes place inside the RC pigment protein, but the impact of quantum effects on this fundamental process remains less clear. Most studies have assumed that this charge separation occurs in the nonadiabatic regime (1,12-19), where coupling between the potential energy surfaces of donor and acceptor states is much weaker than their coupling to the surroundings. However, this nonadiabatic picture is difficult to rationalize with a weak dependence of the rate of P* decay on driving force or temperature, and it has been speculated that charge separation may take place by an adiabatic mechanism arising from strong coupling between the participating P*, $P^+B_A^-$, and $P^+H_A^$ states (20,21). Indeed, following observations of coherent wave-packet motion associated with P* in experiments involving extremely short excitation pulses (10,11,23-27), there have also been indications of coherence in the formation of $P^+B_A^-$ (8,9,28,29) and $P^+H_A^-$ (8,27) that would imply adiabatic electron transfer.

Alternative charge-separation pathways, involving a B_A^* excited state rather than the special pair P* as the electron donor, were observed at low temperature upon direct excitation of B_A (30–32). A fast subpicosecond time constant for electron transfer was found for electron transfer between B_A^* and H_A which is an order of magnitude faster than the 3 ps time constant for electron transfer starting at P* (30–32). In Photosystem II RCs of green plants, this was shown to be the dominant mode of operation at room temperature: the primary radical pair that was formed involved Chl_{D1} (the equivalent of B_A) and H (33,34), whereas at low temperature both pathways of radical pair formation were suggested to be operative (35–37). Whether these reactions occur in the adiabatic or nonadiabatic regime remains to be determined.

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FIGURE 1 Structure and absorbance spectra of the cofactors of the Rba. sphaeroides RC. (a) Space-fill view of the four closely spaced bacteriochlorins that participate in ultrafast charge separation. The two BChls of the P dimer are shown with yellow or gold carbons, the B_A BChl with green carbons, and H_A BPhe with pink carbons. Other atom colors: red, oxygen; blue, nitrogen; magenta, magnesium. (b) Positions of these photoactive bacteriochlorins within the overall arrangement of cofactors, with the route of membrane-spanning charge separation shown by red arrows. The active cofactor branch terminates in a ubiquinone QA (cyan carbons). (c) Absorbance spectrum of the purified *Rba. sphaeroides* RC (*blue*) in the Q_x and Q_y spectral regions. Part of the absorbance spectrum of the carotenoid-deficient R-26 RC is shown in red to emphasize the positions and relative amplitudes of BPhe and BChl absorbance. The excitation pulse (gray) was centered at 880 nm to selectively excite the low-energy transition of the P dimer of BChl. The band at 540 nm corresponds to the Qx transitions of HA and the second RC BPhe, and overlies a broad absorption stemming from carotenoid. The absorption band at 600 nm is attributable to Q_x transitions of B_A, the P dimer, and the second monomeric BChl.

After analyzing electron transfer and protein response in a set of mutants, Wang et al. (38) proposed a novel model of electron transfer that would explain the unusual temperature and driving-force dependence. In this model, the initial charge separation is limited by protein dynamics rather than by a static electron transfer barrier, and conformational diffusion occurs until a configuration with a low activation energy for electron transfer is achieved. Subsequent studies on the wavelength dependence (39) and temperature dependence (40) of electron transfer support this model.

In this study, we aimed to provide a more comprehensive picture of the mechanism of charge separation in the *Rba*. *sphaeroides* RC by carefully measuring the time dependence of the appearance of the anion bands of B_A and H_A during the electron transfer reaction. Previous studies of ultrafast electron transfer in the purple bacterial RC focused mainly on the spectral region between \approx 700 and \approx 950 nm, where the participating bacteriochlorins have the strongest, lowest-energy absorbance bands (Fig. 1 c). However, interpretation of time-resolved difference spectra in this region is complicated by multiple overlapping contributions from ground-state bleaching, anion absorbance, stimulated emission, and electrochromic band shifts. In this work, we reexamined the mechanism of ultrafast charge separation by using broadband pump-probe spectroscopy, focusing on the near-infrared (near-IR) region between 920 and 1060 nm, which contains a well-defined absorbance band for B_A^- (3,41–44) and a less well-studied absorbance band for H_A^- at ≈ 960 nm (7,41,44). Measurements were also made in the visible region between 480 and 710 nm, where H_A has an absorbance band that is well separated from those of P and B_A (Fig. 1 c), and which also contains absorbance bands for B_A^- and H_A^- at ≈ 670 nm.

MATERIALS AND METHODS

Sample preparation

RCs were isolated from *Rba. sphaeroides* using the detergent lauryl dimethylamine oxide (LDAO) and purified using a combination of ion-exchange and gel-filtration chromatography (46). RCs were stored as a concentrated solution in 20 mM Tris-HCl (pH 8.0)/0.1% LDAO and diluted in the same buffer to a concentration of ≈ 0.3 mM for ultrafast spectroscopy.

Pump-probe spectroscopy

The instrument for transient absorption spectroscopy consisted of a 1 kHz laser system, an in-house-built single-path amplified noncollinear optical parametric amplifier (NOPA), and a CCD camera detection system as described previously (47). Briefly, the fundamental pulse was the output of a regenerative Ti:sapphire amplifier operating at 1 kHz (Hurricane; Spectra Physics), producing 85 fs pulses of ≈ 0.6 mJ/pulse centered at 800 nm. This fundamental pulse was split in two parts. One part was chopped one-to-one with a phase-locked chopper operating at 500 Hz for use as a pump for the NOPA to generate 880 nm pulses for excitation. The other part of the fundamental 800 nm output was used to generate a white-light continuum probe pulse in a 2 mm sapphire plate. After passing through the sample, the white-light probe pulses were dispersed in a spectrograph and detected on a 2064 channel CCD array (model S11155-2048; Hamamatsu). The polarization of the probe light was at the magic angle with respect to the pump. The sample was housed in a CaF₂ cell of 120 µm path length, contained in a Lissajous sample scanner that was moved such that a new part of the sample was illuminated at every shot. The spectrum of the probe white-light pulse was modified with different cutoff filters for experiments interrogating either visible and or near-IR spectral regions. Correction for baseline fluctuations of the near-IR time-gated spectra is detailed in the Supporting Material.

The pump pulses were attenuated to an ~40 nJ/pulse and loosely focused to an ≈ 0.4 mm spot size on the sample by adjusting the position of a 40 cm focus length lens. In the probe area of the sample, a photon density of 1.27×10^{14} /cm² was reached, resulting in excitation of only 5% of the RCs. The experiment was performed at room temperature (22°C) and the integrity of the sample was monitored by recording its absorption spectrum before and after each run.

The time delay between pump and probe pulses was controlled by sending the pump beam over a moveable delay line, and absorption changes were collected over time periods of up to 1 ns. To resolve the ultrafast spectral components, time points were taken at 0.05 ps intervals until 3 ps, and at intervals of 0.2 ps until 15 ps. A total of 230 time points were taken over the 1 ns time window, providing sufficient time resolution to resolve every spectral component. Species-associated difference spectra of the participating states were obtained through a target analysis with global fitting of the data.

Global target analysis

We analyzed the broadband femtosecond transient absorption data by globally fitting all of the data simultaneously using a kinetic model (48). This method provided a better description of the system dynamics than can be achieved by fitting to a single time trace. The physical and mathematical basis for global target analysis is that in the data matrix $\Delta A (\lambda, t)$ the variable delay time *t* and wavelength λ are independent, with the measured $\Delta A (\lambda, t)$ being the result of a superposition of several spectral components that can be separated as

$$\Delta A(\lambda,t) = \sum_{i=1}^{N} C_i(t) \Delta \varepsilon_i(\lambda)$$

where $C_i(t)$ and $\Delta \varepsilon_i(\lambda)$ are the concentration and extinction coefficients of component *i*, respectively. We imposed a parametric kinetic model based on a first-order reaction on $C_i(t)$ and extracted the spectral components of $\Delta \varepsilon_i(\lambda)$ by globally fitting the data $\Delta A(\lambda, t)$. $\Delta \varepsilon_i(\lambda)$ is the species' extinction coefficient and may have a different nomination depending on the specific kinetic model used. Based on the kinetic model used, we obtained an expression for $C_i(t)$ by solving the differential equation:

$$\frac{dC_i(t)}{dt} = -k_i C_i(t) + \sum_{j \neq i} k_{ji} C_j(t)$$

where k_i represents the total decay of state *i* and k_{ji} is the microscopic rate from state *j* to state *i*. The reconstructed $C_i(t)$ was convolved with the instrument response function (IRF, Gaussian shape) to globally fit the data by minimizing the expression

$$\sqrt{\left(\Delta A(\lambda,t) - \sum_{i=1}^{N} C_i(t) \Delta \varepsilon_i(\lambda)\right)^2}$$

The fit led to a set of rate constants and the corresponding species spectra $\Delta \varepsilon_i(\lambda)$ simultaneously. The global fitting was performed with the public domain programs TIMP and Glotaran (49) based on the NLLS algorithm.

The model-based global analysis comprises a mathematical description of the dispersion with a third-order polynomial that expresses the wavelength dependence of the maximum of the IRF. We estimated n IRF width of 60–90 fs full width at half-maximum (FWHM) in the visible spectral range and ≈ 80 fs FWHM in the near-IR. The dispersion was ≈ 2.3 ps over the 485–710 nm visible wavelength range, and in the near-IR the dispersion from 920 to 1060 nm was only ≈ 0.3 ps. Effectively, the dispersed traces at 256 wavelengths in the visible range and 1127 wavelengths in the near-IR provided a femtosecond sampling of the ultrafast dynamics. For presentation purposes (cf. Fig. 2), we used the above-determined wavelength dependence of the maximum of the IRF to compute, by interpolation, dispersion-corrected time-gated difference spectra that were reliable after the IRF.

RESULTS

Fig. 2, a and b, show transient spectra in the two spectral regions, and Fig. 2 c shows a selection of time traces at individual wavelengths that emphasize the excellent signal/ noise ratio characteristics of the experimental data.

At 100 fs after excitation of the lowest-energy absorbance band of P at 880 nm, the absorbance difference spectrum recorded showed formation of P*. This was indicated by a very broad and structureless absorbance increase between 470 and 700 nm superimposed on a sharp negative band around 600 nm due to loss of P ground-state absorbance (Fig. 2 a, black), and a strongly sloping negative signal between 920 and 1060 nm arising partly from loss of P ground-state absorbance (maximum at ≈ 870 nm) and partly from stimulated emission from P* (Fig. 2 b, black). Over the next 1.5 ps, a positive band appeared between 630 and 700 nm, consistent with the formation of B_A^- , and a small negative band developed at ≈ 547 nm (Fig. 2 a, cyan). In the 3–12 ps time range, the former narrowed and intensified as the expected H_A⁻ absorbance band formed at ≈ 670 nm (50), whereas the latter intensified with its maximum shifting by ≈ 3 nm to shorter wavelengths. The development of a strong negative band at 544 nm (Fig. 2 a, dark green) is attributable to the loss of H_A ground-state absorbance as $P^+H_A^-$ is formed, and it is notable that this began to grow after only a few hundred femtoseconds (Fig. 2 a, light green and blue).

In the near-IR region (Fig. 2 *b*), a broad absorbance recovery over ≈ 3 ps due to decay of P* stimulated emission



FIGURE 2 Absorption difference spectra after excitation at 880 nm. (*a* and *b*) Dispersioncorrected time-gated absorbance difference spectra between (*a*) 480 and 710 nm, and (*b*) 920 and 1060 nm. (*c*) Time traces at representative wavelengths (rescaled for presentation) on a split linear//logarithmic time axis with fitted kinetics.

was accompanied by the appearance of a B_A^- absorbance band at 1025 nm (3,41–44). This B_A^- absorbance reached a maximum at ~4 ps and then decayed in tandem with the development of an absorbance band with a maximum at 960 nm (see the 12 ps spectrum in Fig. 2 *b*, *dark green*). By comparison with the transient spectra in the visible region in Fig. 2 *a*, this band at 960 nm can be assigned to H_A^- (41), and this band was also reported in spectra of the P⁺H_A⁻ radical pair in previous studies (42,44).

As expected, the experimental data contained spectroscopic signatures of both B_{A}^{-} and H_{A}^{-} during the first few picoseconds of charge separation. To investigate the spectra of the participating states, we carried out a target analysis using a four-component linear scheme with an initial P* state and three subsequent radical pair states: $P^* \rightarrow RP_1 \rightarrow RP_2 \rightarrow RP_3$. This scheme was similar to one that was first proposed by Arlt et al. (3) and is now widely accepted, with inverted kinetics for the first two steps (i.e., the lifetime of the RP₁ intermediate is shorter than that of the preceding P* state). The fitted lifetime of the P* state was 3.3 ps and that of RP1 was 1.2 ps, in line with estimates from the literature (3). Fig. 3 a shows the species-associated difference spectrum (SADS) of each state together with the fitted lifetimes. Representative time traces with fitted kinetics are shown in Fig. S3. The SADS of the first, third, and fourth components had the expected line shapes for P*, $P^+H_A^-$, and $P^+Q_A^-$ (Fig. 3 *a*, black, green, and blue), showing a negative band at ≈ 600 nm due to loss of ground-state absorbance of the P BChls. The SADS of $P^+H_A^-$ (Fig. 3 *a*, green) showed an additional loss of H_A ground-state absorbance at 544 nm and H_A^- absorbance bands at $\approx 665 \text{ nm} (41,50)$ and 960 nm (41,42,44). For the SADS of the RP₁ component, which is expected to correspond to P⁺B_A⁻ (Fig. 3 *a*, *red*), the amplitude of the negative band at $\approx 600 \text{ nm}$ was almost twice that observed in the P⁺H_A⁻ SADS (Fig. 3 *a*, *green*). This was according to expectations, because the ground-state absorbance of both P and B_A at this wavelength is lost in the P⁺B_A⁻ state, but only P absorbance is lost in the P⁺H_A⁻ state (51). In addition, B_A⁻ gave rise to the expected positive band at 1025 nm (Fig. 3 *a*, *red*) (41). Fig. 3 *b* shows the absorption difference spectra of electrochemically reduced BChl and BPheo in solvent for reference with the radical pair protein spectra (reproduced with permission from Fajer et al. (41)).

Although it largely matched expectations, the SADS of \mathbf{RP}_1 had features that were not attributable to either \mathbf{P}^+ or B_A^- (Fig. 3 *a*, *red*). Most obviously, the B_A^- absorbance band at 1025 nm was accompanied by a second positive band at ≈ 950 nm, where absorbance from H_A⁻ is expected (41,42,44) (Fig. 3 a, green). The amplitude of this band was large in comparison with the ${\rm H_A}^-$ band at 960 nm in the $P^+H_A^-$ SADS. The SADS of RP₁ (Fig. 3 *a*, *red*) also contained a negative band around 547 nm. A feature at this position was also seen in a $P^+B_A^-$ SADS in a previous study (19) and assigned to an electrochromic band-shift of the H_A absorbance band. However, in our study, the intensity of this feature was greater than would be expected from a band shift and its shape was more consistent with a partial bleaching of the ground-state absorbance of HA than the S-shaped signal that would be expected for a band shift. Taking these $nonB_A/B_A^-$ features together, we concluded that the SADS of the first radical pair that formed after decay of P* is not



FIGURE 3 Target analysis of absorbance difference spectra. (a) Reaction model with inverted kinetics (top) and SADS obtained from fitting this model to the experimental data. The SADS are color-coded with the reaction model. For presentation purposes, the intensities of the near-IR spectra were multiplied by four in this and subsequent figures. The relative precision of the parameters is better than 5%. (b) Difference spectra H^{-}/H and B⁻/B of bacteriopheophytin and bacteriochlorophyll in solution, reproduced from Fajer et al. (45). (c) Reaction model with reversible electron transfer (top) and SADS extracted from this model. (d) Calculated concentration profiles of each state according to the model in c over a split linear/ logarithmic time axis. The dashed line shows a single exponential decay of 3 ps convoluted with the IRF.

of a pure $P^+B_A^-$ state, but rather represents a mixture of $P^+B_A^-$ and some $P^+H_A^-$.

We investigated other kinetic schemes to determine whether we could obtain a purer SADS for RP₁. Charge separation in RCs is reversible (19,20,52), and therefore rates for reverse reactions were included for the first two steps of the overall reaction scheme (shown in the top right of Fig. 3). Recombination rates reported in pump-probe studies are 4-6 times slower than the forward rates (19,52), but estimates based on observations of delayed emission are often slower (13,20,54). In pump-probe absorption difference data, there is no clean measure for the amount of recombination luminescence, and therefore rates are adjusted such that pure states are obtained (19,52). In this study, introducing a k_{21} rate of $(12 \text{ ps})^{-1}$ for reversal of the first step yielded the spectra shown in Fig. 3 c, and led to a small fraction of long-lived P* (compare the solid line with the dashed line in the concentration profiles in Fig. 3 d). The resulting P^* and RP1 spectra were barely modified by this k₂₁ rate constant, and a much slower value for k_{21} produced similar results. Introducing the rate k_{32} (from RP2 to RP1) had a larger effect, as this led to a small but significant population of RP1 during the lifetime of RP2. Notably, in the analysis with the reversible scheme, the RP1 spectrum gained some RP2 $P^+H_A^-$ signatures, rather than lost them, with a markedly stronger negative band at 547 nm (compare *red spectra* in Fig. 3, *a* and *c*). The failure of the reversible model to yield pure RP₁ spectra can be explained by the fact that the H_A^- features, though similar to those of H anion in solvent, were markedly different from those of RP2 $P^+H_A^-$: the bands around 545 nm and 950 nm showed a red and blue shift, respectively, as compared with the later RP2 $P^+H_A^-$ spectrum. We conclude that including reversibility into the first two steps of charge separation did not remove the contribution of H_A^- to the SADS of RP₁.

As an alternative, we applied a target model in which the second step of electron transfer was slower than the first, as proposed recently (44). This produced a physically unreasonable lifetime for P* and also an incorrect SADS for RP₁ in the visible region (see Fig. S4). This possibility was therefore rejected.

Next, we considered the possibility that a more complex model could yield RP₁ spectra lacking H_A^- features. Such a model should account for the following observations: 1), a very fast formation of H_A^- , to account for the presence of H_A^- features at early times; 2), a long-lived B_A^- resulting in a significant concentration of B_A^- on the picosecond timescale, to reduce the amplitude of the RP₁ spectrum to a level consistent with the RP₂ spectrum; and 3), a P* lifetime of ~3 ps. We could not account for these observations using a homogeneous reaction scheme, but were able to do so by using a heterogeneous scheme employing two branches with charge separation rates that were allowed to vary freely. Fig. 4, *a* and *b*, show the concentration profiles



FIGURE 4 Target analysis of absorbance difference spectra with heterogeneous fast and slow populations, both exhibiting inverted kinetics. (*a*) Reaction model (*top*) and populations of the intermediates over a split linear/logarithmic time axis. Note that for $P^+Q_A^-$, the two populations have been added. The relative precision of the parameters is better than 10%. (*b*) Estimated SADS, color-coded with the reaction model. Note that the black and gray SADS are identical, as are the orange and red SADS.

and the SADS obtained with two parallel reaction schemes, again of the form $P^* \rightarrow RP_1 \rightarrow RP_2 \rightarrow RP_3$. The optimal fit resulted in a 72% fast fraction with a time constant of 1.9 ps for $P^* \rightarrow RP_1$, 1.0 ps for $RP_1 \rightarrow RP_2$, and 172 ps for $RP_2 \rightarrow$ RP_3 , and a 28% slow fraction with equivalent time constants of 4.6 ps, 3.6 ps, and 446 ps respectively. The SADS of RP_1 (Fig. 4 *b*, *red*) now contained only a B_A^- absorbance band at 1025 nm, with an amplitude comparable to that of H_A^- in the RP₂ SADS. In the visible region, a small feature in the SADS of RP₁ at ~545 nm was more clearly an S-shaped band shift attributable to an electrochromic effect on H_A rather than a negative feature attributable to a H_A reduction. We note that because in all cases the final spectrum is that of $P^+Q_A^-$, the slower RCs are also open (i.e., with Q_A oxidized) and fully functional.

To investigate whether this heterogeneity could be seen as an effective description of what may in reality be a distribution of rates, we fitted a homogeneous model with a Gaussian distribution of the first two rates to the near-IR part of the data set. This resulted in decay-associated difference spectra (DADS) consistent with the spectra in Fig. 4. The rate associated with the decay of P* was centered at 0.4 ps^{-1} , and the rate associated with the decay of B_A⁻ at 0.7 ps^{-1} . A more detailed description of this modeling is given in the Supporting Material.

The heterogeneous model was further tested with data measured in the Q_x region after excitation of H at 743 nm. A target analysis using the inverted kinetics scheme resulted in the SADS depicted in Fig. S5 A. The quality of these data and fit is demonstrated in Fig. S6. The first SADS (magenta) shows the expected features of H*, with bleach near 540 nm superimposed upon a broad excited-state absorption. After 0.24 ps, H* transfers its energy to P*. The SADS of the second, fourth, and fifth components had the expected line shapes for P*, $P^+H_A^-$, and $P^+Q_A^-$ (Fig. S5 A, black, green, and blue). Again, the SADS of RP1 with the inverted kinetics scheme resembled $P^+[B_AH_A]^-$. The heterogeneous target shown in Fig. S5 B resulted in an RP₁ SADS with only a small band-shift feature around 545 nm. The small differences between the SADS of Fig. S5 B and Fig. 4 b are attributed to differences in the overlap of the pump and probe beams in the two experiments.

DISCUSSION

The experimental data outlined in Fig. 2 demonstrate a reduction of the secondary electron acceptor HA on an unexpectedly fast timescale. We applied several kinetic models to the data to investigate the origins of this finding, and below we discuss the physical implications of these models. First, however, it is relevant to consider whether alternative charge-separation pathways could be responsible for the experimental observations. Alternative charge-separation pathways, involving BA* as the electron donor rather than P*, have been observed at low temperature upon excitation of B_A, and a fast subpicosecond time constant for electron transfer was reported for electron transfer between B_A and H_A (30–32). In our experiments, we excited RCs at 880 nm to minimize any excitation of B_A, but in principle, excitation energy transfer between P and B_A could lead to a small equilibrium fraction of BA*. If electron transfer from BA* is also ultrafast at room temperature, this could lead to a small fraction of charge separation occurring by a mechanism that bypasses P*. However, the spectrum resolved for P* showed no contamination with features indicating a radical pair, and therefore it seems reasonable to conclude that this process does not play an important role in charge separation in RCs at room temperature when P is excited directly on the low-energy side of its lowestenergy absorption band.

Adiabatic electron transfer

The homogeneous inverted-kinetics models, both with and without reversible reaction kinetics (Fig. 3, *top*), yield a difference spectrum for RP₁ with features indicative of the formation of P⁺, B_A⁻, and H_A⁻. This raises the question as to what mechanism could account for the first radical pair formed after P* decay not being a pure P⁺B_A⁻ state, but rather containing a sizeable contribution from a P⁺H_A⁻ state that has spectral features slightly different from the later, relaxed P⁺H_A⁻ state.

Electron transfer in biological systems is usually discussed in terms of a nonadiabatic process. Descriptions such as that developed by Marcus and Sutin (18) assume that the electronic coupling between the reactant and product states for each step is much weaker than coupling to their surroundings, and that vibrational cooling is much faster than electron transfer. A schematic representation of primary charge separation as intersecting potential energy surfaces for the diabatic states P^* , $P^+B_A^-$, and $P^+H_A^-$ is shown in Fig. 5 a. However, a requirement for nonadiabatic electron transfer, i.e., that vibrational relaxation must take place before electron transfer, has been shown to not be fulfilled for the bacterial RC. Coherent motion of a vibrational wave packet in the P* state created using an ultrashort (<30 fs) excitation pulse persists for at least 1 ps, even at room temperature (10,11,56). In this work, the IRF was 60-90 fs FWHM, which probably prevented us from observing such oscillations. The two limiting regimes in electron transfer, adiabatic and diabatic transfer, are commonly defined according to the value of the hopping probability P_h . If $P_h \approx 1$, the reaction is said to be adiabatic. Each time the molecular system reaches the seam region, the probability to hop to the other quantum state is nearly



FIGURE 5 Potential energy surfaces for participating states under weak and strong coupling regimes. (*a*) Weak coupling, nonadiabatic electron transfer. The system moves on the potential surface of the initial state (*red broken arrow*) before hopping with a low probability to the intersecting potential surface of the first acceptor state (*red solid arrow*), followed by a low-probability hop to the second acceptor (not shown). (*b*) Strong coupling, adiabatic electron transfer. After electron injection from P* in 3 ps, the system moves in a coherent manner along a continuous adiabatic potential surface formed by the P⁺B_A⁻ and P⁺H_A⁻

states (*red arrow*). Fast hopping of the electron between B_A and H_A in combination with the slow, inhomogeneous 3-ps electron injection leads to the observation of a time-averaged $P^+[B_AH_A]^-$ spectrum. This state relaxes with a time constant of 1.2 ps to the state $P^+H_A^-$.

one. On the other hand, the hopping probability may be rather small ($P_h \ll 0.1$), and then the reaction is qualified as nonadiabatic. The magnitude of the hopping probability transition is dependent on the electronic coupling between two states and on the decoherence time (57).

The observation that the first radical pair has a mixed $P^+B_A^-/P^+H_A^-$ character raises the question as to whether electronic coupling between BA and HA is sufficient to place the $P^+B_A^- \rightarrow P^+H_A^-$ electron transfer in the adiabatic regime. To address this issue, the coupling relative to the decoherence time and the energy gap between H and B needs to be considered. Energy transfer between the P, H, and B cofactors occurs extremely rapidly, from H to B in ~100 fs and from B to P in ~150 fs (24,56,58-60), and occurs on the same timescale as electronic dephasing of both H and B (60-90 fs at room temperature) (56,58), indicating a strong electronic coupling between B and H. Dephasing of coherence between the two electronic states that are formed by mixing of the H* and B* excited states has been observed to be very slow (310 fs at 180 K), implying that transition energy fluctuations on adjacent B and H cofactors are strongly correlated (4). In combination with an estimated electronic coupling strength of 220 cm^{-1} relative to the energy gap between excitonic H and B states (4) of 680 cm^{-1} , this has been proposed to be sufficient to put the reaction in an adiabatic regime (57).

Given these considerations, it is possible that the presence of the spectroscopic signatures for both B_A^- and H_A^- in the SADS of the first radical pair product state is a consequence of photochemical charge separation in the Rba. sphaeroides RC occurring by an adiabatic mechanism (Fig. 5 b). In this adiabatic picture, after photoexcitation of P and electron donation by P*, the electronic wave packet moves coherently on an adiabatic surface formed by strongly coupled $P^+B_A^-$ and $P^+H_A^-$ states. In an analysis, one would not resolve the actual adiabatic electron transfer between BA and H_A, but rather observe the time-averaged mixture of these states. The relatively slow (3 ps) feeding of such a state from P* may intrinsically hamper a more direct observation of wave-packet motions over the coupled $P^+B_A^-$ and $P^+H_A^-$ potential surfaces (61). Subsequent vibrational cooling leads to localization of the electron on H_A in ~1 ps. Decay of P* therefore initially produces a state that is a mixture of vibrationally hot $P^+B_A^-$ and $P^+H_A^-$ states, which then relaxes into the cool $P^+H_A^-$ state.

An additional observation from the data is that RP_1 appears to have a very broad and intense absorption throughout the spectral regions investigated, in addition to positive anion bands at 950 nm (H_A^-), 1025 nm (B_A^-), and 670 nm (B_A^- and H_A^-) (Fig. 3 *a*, red). Considering that the electron is delocalized over a larger range of cofactors than in the other states, this continuum-like transition can be attributed to the larger polarizability of the electronic cloud of the donor-acceptor complex (62–64). However, a large amplitude of a short-lived spectrum may also be an

indication that the model is not sufficient to describe the data, and that more advanced modeling needs to be applied.

Heterogeneity in electron transfer rates

The separation of RCs into a fast and a slow fraction, each following a simple reaction scheme, yielded SADS consistent with the sequential transfer of the electron from P* to B_A and from B_A to H_A. The fast (2 ps, 1 ps, 172 ps) and slow (4.6 ps, 4 ps, 446 ps) fractions could be a consequence of the dynamic protein environment causing distributions in electronic coupling, reorganization energy; and/or activation energy, creating ensembles of RCs that perform very fast charge separation and ensembles that perform more slowly. Such environmental variations could have a very local origin, such as the temporal absence or presence of a hydrogen bond, or could be due to more global variations in protein structure. However, it may also be that we have described here two extreme situations that actually arise from the convolution of two processes, each of which has a distribution of rates. With moderate widths centered at 0.4 ps^{-1} and 0.7 ps^{-1} , an equally good description of the data can be obtained. At present, it is difficult to distinguish between these two situations. In view of the reported important role of conformational changes that optimize the electron transfer pathway (38-40), the fast rate could be interpreted as the intrinsic electron transfer rate in an optimized configuration, with the slower rate delayed by the typical time it takes for the protein to reach a conformation optimal for electron transfer. Heterogeneity in BRC electron transfer rates has been reported before. Kirmaier and Holten (65) reported that some fractions of the RCs carry out electron transfer at a fast rate of $(1.3 \text{ ps})^{-1}$ and $(100 \text{ ps})^{-1}$, and some do so at a comparatively slower rate of $(4 \text{ ps})^{-1}$ and $(300 \text{ ps})^{-1}$ for the electron transfer from P to H_A and H_A to Q_A, respectively. Here, we show that fast electron transfers are even further correlated with fast electron transfer from P to B_A and from B_A to H_A. This correlation would suggest that protein conformational changes that convert RCs from a fast conformation into a slow one, and vice versa, occur on a timescale slower than electron transfer (i.e., > 1 ns), and consist of a large-scale conformational change such as expansion and compression movements of the full protein. Conformational changes in the BRC have been reported to occur near both QA and QB (66-70), and near the special pair P, where the side chain of TyrL162 was observed to move closer to P after photoactivation (71). There are strong indications that the Q_A^- to Q_B electron transfer is gated (66-69) by conformational changes suggested to be triggered by the formation of Q_A^{-} (70). It is possible that either the slow or fast fraction of RCs that we observed here is due to the reexcitation of RCs within the lifetime of these conformational changes, which may be >20 min (72). In contrast to the study by Kirmaier and Holten (65), we observed no indications of spectral differences between RCs that perform fast or slow electron transfer.

The basic observation that we report here is that when the commonly accepted sequential electron transfer scheme with inverted kinetics and 3 ps and 1 ps time constants is applied, the SADS resolved for RP1 is a mixture of B_A^- and H_A^- . This mixture can be interpreted either statically, with some RCs having very short-lived B_A^- and making very fast H_A^- , or dynamically with an electron traveling back and forth between B and H, such that a time-averaged mixture is observed. Experiments performed as a function of temperature to enhance the effect of heterogeneity or as a function of reexcitation delay, or in which the B and H anion bands are pumped selectively to probe the coupling within $P^+[B_AH_A]^-$ may provide more insight into the mechanism of charge separation.

CONCLUSIONS

In this work, we observed the unexpected involvement of the H anion in the electron transfer state usually identified as $P^+B_A^-$, demonstrating that charge separation in the bacterial RC has aspects that are still not fully understood. Several models were examined to account for this observation. In light of the electronic coupling strength between B and H, and the ultrafast timescale involved, the presence of unrelaxed $P^+H_A^-$ features mixed with $P^+B_A^-$ in the first radical pair state may be the result of an adiabatic step in the BA to HA electron transfer in bacterial RCs. Alternatively, the presence of an $\mathrm{H_A}^-$ anion band in the near-IR can be interpreted as arising from a subtle heterogeneity in the electron transfer process, in which most RCs are in a conformational state that facilitates fast two-step electron transfer in \approx 2 ps and 1 ps hops, followed by H_A to Q_A electron transfer in 172 ps, whereas others take 4 ps or more per hop from P via B_A to H_A, and 446 ps for the H_A to Q_A electron transfer.

SUPPORTING MATERIAL

Six figures and additional analysis are available at http://www.biophysj.org/ biophysj/supplemental/S0006-3495(13)00459-1.

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Supporting Information

Early bacteriopheophytin reduction in charge separation in reaction

centers of Rhodobacter sphaeroides

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Correction for baseline fluctuations in the near-IR data.

The matrix of the residuals of the near-IR data is highly structured due to baseline fluctuations. This structure is revealed by a singular value decomposition of the matrix of the residuals (48). The screenplot with the logarithm of the singular values, Figure S1E indicates that the first singular value of the matrix of the residuals stands out, and is responsible for a large part of the rms error of the fit. The first right singular vector (Figure S1D) consists of a constant term plus a wiggle, and represents the baseline fluctuations, noise that is highly correlated in the time gated spectra. The first left singular vector (Figure S1C) is unstructured, indicating that there is no correlation in the noise between subsequent spectra. We therefore subtracted these estimated baseline fluctuations from the data, thereby reducing the rms error of the fit from 0.450 mOD to 0.203 mOD. Representative traces at 960 and 1025 nm before and after the correction for baseline fluctuations are depicted in Figure S1A,B (before) and Figure S1F,G (after).



Figure S1. Representative traces at 960 and 1025 nm before (A,B) and after (F,G) the correction for baseline fluctuations. Red lines indicate global fits using four lifetimes. Note that the time axis is linear until 1 ps, and logarithmic thereafter. Insets show residuals. Results from Singular Value Decomposition of the matrix of the residuals: first left (C) and right (D) singular vector, and screeplot with the logarithm of the singular values.

Distributed fit of the near IR data

A global fit using four monoexponential decays results in the four decay associated difference spectra (DADS) of Figure S2B. The two fastest estimated lifetimes are 1.5 and 3.4 ps. The third lifetime was fixed at 200 ps, in view of the visible region data where this value was estimated. The fourth lifetime was much larger than the 1 ns measurement window. The fourth DADS (light green) can be interpreted as P^+ , the third DADS can be interpreted as H_A^- . The second DADS (red) can be interpreted as decay of P* (stimulated emission above 920 nm) in combination with rise of the B_A^- band around 1025 nm. Because of the inverted kinetics this rise is a positive feature in the red DADS, and a negative feature in the black DADS (1). The broad negative feature in the black DADS below 990 nm is more difficult to assign. In combination with the 920-955 nm positive feature superimposed on the red 3.4 ps DADS it represents early $P^+H_A^-$.

When the fastest two decays are assumed to be normally distributed (2) on a logarithmic

scale, $\int_{-\infty}^{\infty} \exp(-(\log(k) - \log(k_c))^2 / (2\sigma^2)) \{\exp(-kt) \otimes IRF(t)\} d \log(k)$, the distributions depicted in Figure S2E are estimated. The shapes of the red, blue and green DADS in Figure S2D and B are identical, but the black DADS is different below 990 nm, due to the "faster decay part" of the second component. The rms error of the fit is only slightly better (0.1%), indicating that the data are consistent with the fast decays being monoexponential or distributed. For target analysis the distributions of the two fast decays can be mimicked by a heterogeneous model with a linear combination of exponentials for the formation of the P⁺B_A⁻ and P⁺H_A⁻ states. From the overlap of the black and red distributions we infer that there are two extremes for the formation of the P⁺B_A⁻ and P⁺H_A⁻ states: fast – fast, resulting in early P⁺H_A⁻, and slow – slow resulting in the ≈ 3.4 ps P* decay and an appreciable transient population of P⁺B_A⁻.



Figure S2. Global analysis of the near IR data using four monoexponential decays (A,B) or two distributed decays and two monoexponential decays (C,D,E). Key: black 1.5 ps (A) and k_c =0.7/ps (C), red 3.4 ps and k_c =0.4/ps (C), blue 200 ps, green long lived. Time axis in (A,C) is linear until 1 ps, and logarithmic thereafter.



Figure S3. Time decay traces after excitation of P at representative wavelengths (ordinate labels) of data (black) and fit (red) according to the heterogeneous kinetics model as in Figure 4. Time axis is linear until 1 ps, and logarithmic thereafter. Spikes straddling time zero are attributed to coherent artifact.



Figure S 4. Result of global fitting based on a sequential scheme with increasing lifetimes. (A) Model, fitted SADS and time constants. (B) Fitted populations of each state according to the model in (A). Although this model yielded a better separation of the species RP₁ and $P^+H_A^-$ in the NIR region there were three reasons to discard it: 1) a clear contribution from P* emission was still present at 960 nm in this SADS of the RP₁ state; 2) the lifetime of P* was too short to match the observed P* fluorescence life time of \approx 3ps (represented by the dashed line in panel B); 3) the intensities of the bleach at around 600 nm for RP₁ and P⁺H_A⁻ were not consistent with expectations. Kirmaier et al (*3*) have shown that the bleach at 600 nm for P⁺B_A⁻ is larger than for P⁺H_A⁻ by comparing the spectroscopic differences between wild-type RCs and a L214H mutant in which the H_A BPhe is replaced by a BChl.



Figure S 5. Target analysis of absorbance difference spectra after excitation of H. (A) Reaction model with inverted kinetics (top) and SADS obtained from fitting this model to the experimental data. The SADS are colour coded with the reaction model. (B) Reaction model with heterogeneous fast and slow populations, both exhibiting inverted kinetics and estimated SADS.



Figure S 6. Time decay traces after excitation of H at representative wavelengths (ordinate labels) of data (black) and fit (red) according to the heterogeneous kinetics model as in Figure S 5. Time axis is linear until 1 ps, and logarithmic thereafter. Spikes straddling time zero are attributed to coherent artifact.

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