Biochimica et Biophysica Acta, 1017 (1990) 112–117
Elsevier

BBABIO 43213

Relative phototrapping rates of the two bacteriopheophytins in the photoreaction center of *Ectothiorhodospira* sp.

Ted Mar and Gabriel Gingras

Département de biochimie, Université de Montréal, Montréal (Canada)

(Received 5 September 1989)
(Revised manuscript received 27 December 1989)

Key words: Bacteriopheophytin; Phototrapping; Reduced intermediate; Reaction center; Cytochrome *c*; (*Ectothiorhodospira* sp.)

The photoreaction center of *Ectothiorhodospira* sp. includes a bound *c*-type cytochrome containing four hemes. When reduced, this cytochrome competes with the reduced primary electron acceptor for charge recombination with the oxidized primary donor. This allows the phototrapping of reduced intermediary species. While only the A arm bacteriopheophytin anion was phototrapped at temperatures above 250 K, both bacteriopheophytin anions were phototrapped at 219 K. The trapping and decay rate constants of the two bacteriopheophytin anions were obtained from the kinetics were obtained from the kinetics of their light-induced absorbance change and EPR signal. The bacteriopheophytin situated in the A arm was phototrapped 274-times faster than the one in the B arm. Both phototrapped anions decay by charge recombination with the oxidized cytochrome.

Introduction

The X-ray structure analysis of the photoreaction centers of *Rhodopseudomonas* (*Rp.*) *viridis* [1] and *Rhodobacter* (*Rb.*) *sphaeroides* [2,3] have shown that the four bacteriochlorophyll (Bchl) and the two bacteriopheophytin (BPh) molecules are arranged with a C_2 symmetry. This arrangement suggests two symmetric electron transfer pathways branching from the Bchl special pair (P), each pathway comprising one Bchl and one BPh. The two BPh molecules are differentiated by the position of their Q_x band absorption peaks [4]. One is red-shifted to 450 nm (Φ_A) while the other is blue-shifted to 530 nm (Φ_B). Picosecond laser flash spectroscopy has shown that the initial charge separation occurs, within 2.8 ps, between P and Φ_A, the red-shifted BPh [5]. This technique has provided no indication of the reduction of Φ_B, the blue-shifted BPh [6,7]. The preference of electron transfer for one of the two symmetric electron transfer pathways (A branch) implies a structural asymmetry. Plato et al. [8] using detailed X-ray structural data and magnetic resonance have theoretically calculated the rate of electron transfer along the A branch to be 34-times greater than that along the B branch. However, such predictions as those of Plato et al. [8] as well as future theoretical calculations demand an accurate experimental determination of the ratio of the asymmetric electron transfer rate constants.

So far, this has not been precisely measured because the picosecond flash technique is not sensitive enough to measure the low quantum yield electron transfer along the B pathway [9]. In this study, we have used the phototrapping technique [10–13] to measure the photoreduction kinetics of both Φ_A and Φ_B and found that Φ_A is phototrapped 274-times faster than Φ_B.

Materials and Methods

Ectothiorhodospira sp. (ATCC 31751) was cultured in 13 liter cylindrical bottles as described before [14]. Photoreaction center, prepared according to Lefebvre et al. [14], was suspended in glycerol/100 mM Tris-HCl/Triton X-100 (1 : 1 : 0.01, v/v) (pH 7.4) containing 12 mg of sodium dithionite per ml of suspension. The samples were degassed by evacuation and placed in sealed Infrasil cuvettes 3 mm^2 in section.

Absorbance changes were measured with a Cary 14 R spectrophotometer equipped with an actinic light to permit cross-illumination of the sample. The actinic light was provided by a 650 W tungsten-halogen lamp filtered through a Baird Atomic interference filter. The transmittance curve of the filter is shown in Fig. 1 (dotted line). The sample was placed in an Air Products Joule-Thomson cryostat cooled by nitrogen gas.

Electron paramagnetic resonance measurements were carried out with a Varian E-104 A spectrometer operat-

Correspondence: T. Mar, Département de biochimie, Université de Montréal, Montréal, Québec, Canada, H3C 3J7.

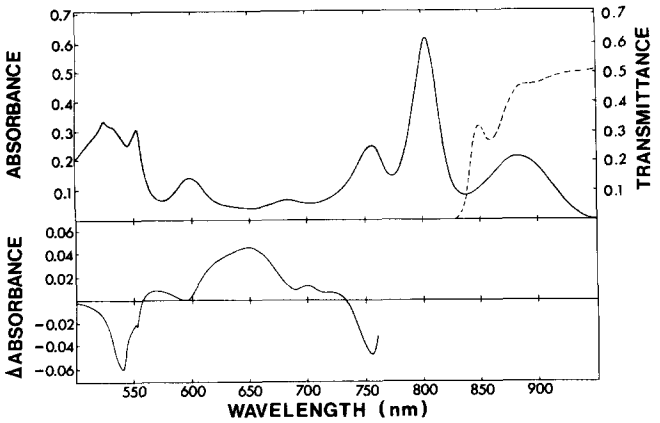

Fig. 1. Absorption spectrum (upper panel, smooth curve) and light-induced difference spectrum (lower panel) of *Ectothiorhodospira* sp. photoreaction center. The sample was suspended in a solution of glycerol (1 : 1, v/v) and 100 mM Tris-HCl/0.1% Triton X-100 containing 12 mg of sodium dithionite per ml of suspension. The absorption spectrum was determined at room temperature. The difference spectrum (lower panel) was obtained by illuminating the sample at 265 K by a tungsten lamp filtered with a Baird Atomic 930 wide band interference filter whose transmittance spectrum is shown (dotted line) in the upper panel.

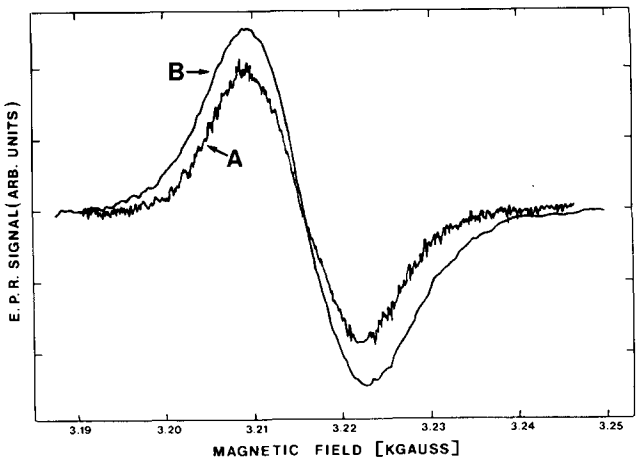

Fig. 2. EPR spectra of photoreaction center from *Ectothiorhodospira* sp. induced by illumination at 158 K (curve A) and at 219 K (curve B). In curve A, the spectrum was recorded 1 min after illumination by a 3 s. light pulse. In curve B, the spectrum was recorded under continuous illumination. Illumination conditions as in the legend of Fig. 6. The spectra were recorded with a modulation frequency of 100 kHz with microwave power of 1 mW and a modulation amplitude of 4 G. The sample was suspended in a solution of glycerol (1 : 1, v/v) and 100 mM Tris-HCl/0.1% Triton X-100 containing 12 mg of sodium dithionite per ml of suspension.

ing at 9.01 GHz in an E231 cavity. The samples were placed in a Varian E257 variable temperature accessory.

The risetime, T, of phototrapping was obtained from the kinetic curves of the photo-induced change in absorbance and EPR signal after correction for the instrumental response time. The measured kinetic curves were fitted with theoretical curves $F(t)$ calculated from the equations were

$$F(t) = \int_0^t G(u)H(t-u)\,du$$

$$G(u) = (G_M/T)\exp-(u/T)$$

$$H(t-u) = 1-\exp-(t-u/t_i)$$

and where G_M is the change observed after a prolonged illumination; t_i is the instrument response time as determined from the measured photo-induced P^+ rise kinetics; t is the duration of illumination.

Results

Trapping of Φ_A^-

Illuminating P890 of the Bchl special pair at 265 K in the presence of dithionite results in the bleaching of the Φ_A absorption bands at 540 and 755 nm and in the concomitant appearance of a broad absorption band centered at 650 nm (Fig. 1). Because the absorbance increase in the 650 nm region is indicative of a pi-anion radical [15], the bleaching of the BPh Q_x and Q_y absorption bands may be attributed to the monoelectronic reduction of Φ_A. In support of this, the unpaired spin of Φ_A^- was also detected by electron spin resonance. Photoreaction centers illuminated in the presence of dithionite at 158 K showed an EPR signal with a g value of 2.0039 and a peak to peak linewidth of 13 G (Fig. 2, curve A). This spectrum was recorded at 158 K, approx. 1 min after a 3 s light-pulse as discussed below. This signal is characteristic of monomeric BPh anion in vitro [15] and is similar to that reported for other systems [16–18].

Fig. 3 shows the phototrapping kinetics of Φ_A^- as determined by the photo-induced ΔA_{645} at 265 K. Upon illumination, the ΔA_{645} rapidly increased according to a single exponential rate. On turning off the light,

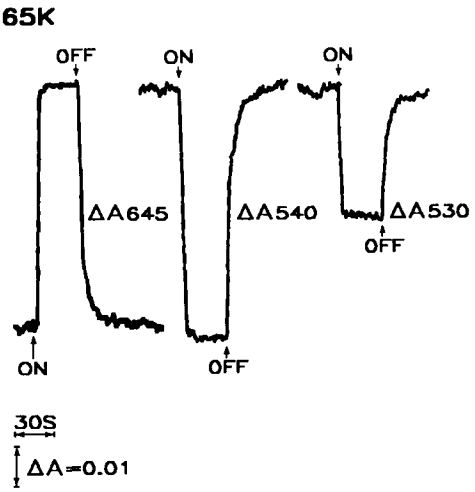

Fig. 3. Kinetics of light-induced absorbance change at 645, 540 and 530 nm of *Ectothiorhodospira* photoreaction center measured at 265 K. Experimental conditions as in Fig. 1.

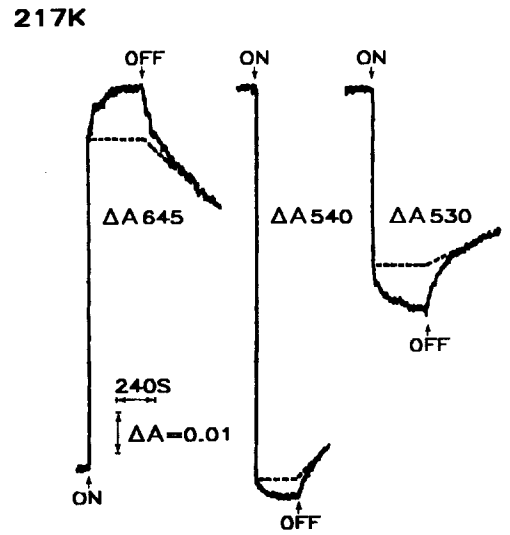

Fig. 4. Kinetics of light-induced absorbance change at 645, 540 and 530 nm of *Ectothiorhodospira* sp. photoreaction center measured at 217 K. Experimental conditions as in Fig. 1. The kinetic curve is resolved into a slow and a fast-rising component (see text) the latter of which is shown by the dotted curve.

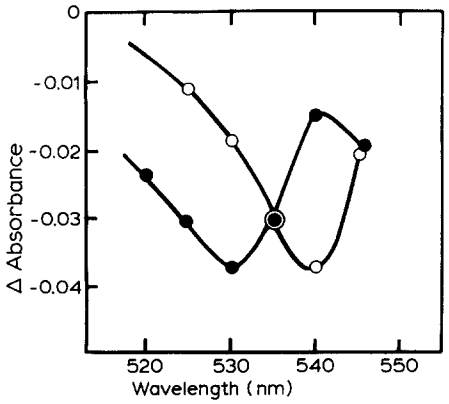

Fig. 5. Difference spectra of the two kinetic components of the light-induced absorbance change in *Ectothiorhodospira* sp. photoreaction center at 222 K. The absorbance change was measured after 5 min of illumination. The slow-rising component (●) was normalized at the band peak of the fast-rising component (○). Experimental conditions as in Fig. 1.

the absorbance decayed back to its original value with a single exponential time constant of 7 s. The single exponential decay is consistent with the trapping of only one BPh^-. Similarly, the ΔA_{530} and the ΔA_{540} have only one kinetic component, ΔA_{540} being larger than ΔA_{530}. At this temperature, therefore, only Φ_A^- was trapped. Lowering the temperature to 250 K increased the monophasic decay time constant to 28.9 s (not shown). The decay time of the trapped Φ_A^- anion in *Ectothiorhodospira* sp. photoreaction center is noticeably short compared with that found in *Rps. viridis* [16] and in *Rb. sphaeroides* [18] (half decay times of 10–15 min at room temperature).

Trapping of Φ_B^-

Lowering the temperature to 217 K (Fig. 4) caused the light-induced ΔA_{645}, ΔA_{540} and ΔA_{530} rise and decay kinetics to become biphasic, with the appearance of a slow component. On turning off the light, the decay kinetics at all three monitoring wavelengths showed a fast component as if the new slow-rise component decayed more quickly than the fast-rise component. This additional component, characterized by a positive ΔA_{645} and corresponding negative ΔA_{540}, ΔA_{530} (Fig. 4) and ΔA_{755} (not shown) is indicative of the photoreduction of another species, presumably Φ_B. The fact that the two BPhs have different Q_x absorption peaks offers a means of monitoring their respective phototrapping rates. While the slow-rising component is more prominent at 530 nm than at 540 nm, the faster component, as also observed at 265 K (Fig. 3), is greater at 540 nm than at 530 nm. The spectra of the slow-rising and fast-rising components (Fig. 5), obtained by varying the monitoring wavelength, have peak absorbance changes at 530 nm and at 540 nm, respectively. These results indicate that the new trapped species is the blue-shifted Φ_B^-. Whereas Φ_A^- is trapped quickly and has a long lifetime at low temperatures, Φ_B^- is trapped much more slowly but has a shorter lifetime.

If the assignment of the new trapped species to anionic Φ_B^- is correct, a new EPR signal should be observed. A continuous illumination at 219 K in the

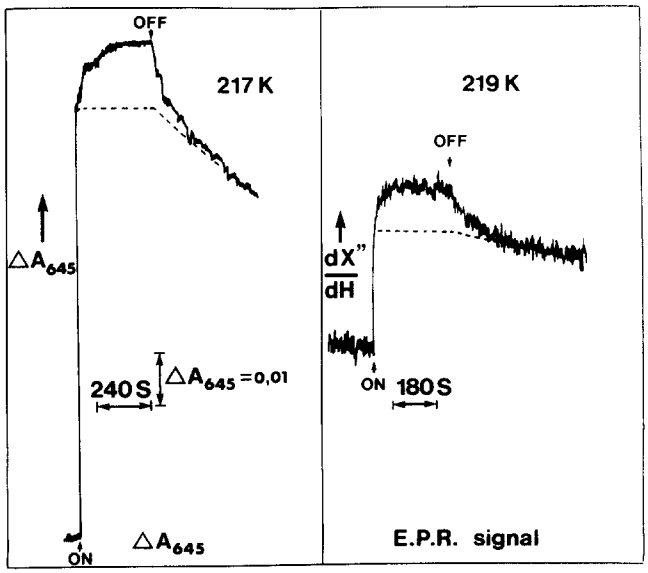

Fig. 6. Kinetics of the light-induced 645 nm absorbance change measured at 217 K (left panel) and of the light-induced EPR signal measured at 219 K (right panel). The sample was suspended in a solution of glycerol (1:1, v/v) and 100 mM Tris-HCl/0.1% Triton X-100 containing 12 mg of sodium dithionite per ml of suspension. The EPR signal was recorded at a set magnetic field at a microwave frequency of 9.01 GHz and microwave power of 1 mW. The sample was illuminated by white light provided by a 500 W tungsten lamp. The light was focused through a 1 cm diameter light guide placed at an opening on the side of the sample cavity.

presence of dithionite results in an EPR signal with a g value of 2.0039 and peak-to-peak linewidth of 13 G (Fig. 2, curve A). Like that of the ΔA_{645} signal (Fig. 6, left panel), the kinetics of this EPR signal measured at a set magnetic field (Fig. 6, right panel) is biphasic, consisting of a slowly rising component with a short lifetime and of a fast rising component with a long lifetime. The biphasic properties of this signal were used to kinetically resolve the spectrum of Φ_A^- and Φ_B^-. The EPR spectrum of Φ_A^-, as reported above, was measured at 158 K (at this temperature, the signal due to the dithionite anion becomes too broad to interfere) following a 3 s light pulse (Fig. 2, curve B). At 158 K, Φ_A^- has a very long lifetime and a 3 s light pulse is long enough to trap Φ_A^- but is too short to trap much Φ_B^-. To ensure that the EPR spectrum was that of Φ_A^-, it was recorded approx. 1 min following illumination to allow the decay of any possibly trapped Φ_B^-. The EPR spectrum obtained after a continuous illumination (Fig. 2, curve B) is attributed to a mixture of Φ_A^- and Φ_B^-. Since the shape of this spectrum is identical to that attributed to Φ_A^- (Fig. 2, curve A), we cannot distinguish, except kinetically, between the EPR signals of Φ_A^- or Φ_B^-.

Comparison of the phototrapping rates of Φ_A^- and Φ_B^-

The rate constants for the phototrapping of Φ_A^- and Φ_B^- can be determined from the kinetic curves of Figs. 3, 4 and 6. The absorbance changes as a function of time were fitted with the best theoretical curve calculated by the equations:

$$\Delta A(t) = \Delta A_A[1-\exp-(k_A t)] + \Delta A_B[1-\exp-(k_B t)],$$

$$for\ 0 < t < t_0 \qquad (1)$$

$$\Delta A(t-t_0) = \Delta A_A \exp-(k_A^{-1}(t-t_0)) + \Delta A_B \exp-(k_B^{-1}(t-t_0)),$$

$$for\ t > t_0 \qquad (2)$$

where ΔA_A and ΔA_B are the maximum measured change in absorbance of components A and B, respectively; variable t is the time elapsed after the onset of illumination and t_o is the value of t when the light is turned off; k_A and k_B are the phototrapping rate constants and k_A^{-1} and k_B^{-1} are the decay rate constants of the two components. Similarly, the change in the EPR signal with time of illumination (Fig. 6, right panel) was fitted with the best theoretical curve calculated with Eqns. 1 and 2. The parameters ΔA_A, ΔA_B, k_B, k_A^{-1} and k_B^{-1} were found by best fit to the experimental curves in Figs. 4, 6 and 7. k_A was found by measuring the change in absorbance and EPR signal at a faster time-scale (not shown) and was corrected for instrumental response time (see Materials and Methods). The k_A and k_B values are expectedly different for optical and for EPR measurements because of the unmatched absorbed light intensities in the two types of measurement due to sample concentration and geometry. However, as shown below, their ratio, k_A/k_B, is the same for optical and for EPR measurements.

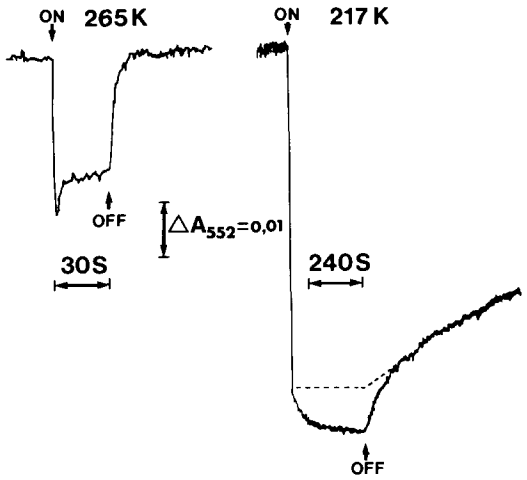

Fig. 7. Kinetics of the light-induced cytochrome c absorbance change at 552 nm in *Ectothiorhodospira* sp. photoreaction center at 265 K and 217 K. Experimental conditions are as in Fig. 1. The dotted curve is the fast-rising component resolved from the measured kinetic curve (see text).

ΔA_B is small compared with ΔA_A because the decay rate constant k_B^{-1} is comparable to the phototrapping rate constant k_B. Conversely, the amount of Φ_A^- trapped under steady-state conditions is large because the rate of decay is much slower than the rate of phototrapping. The measurement of the phototrapping rate constant is also affected by a large decay rate constant. The true phototrapping rate constant and absorbance change can be obtained by correcting the measured values for the effect of the decay rate constant. The process of phototrapping a species I^- can be written simply as

$$[I] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [I^-]_t$$

where k_1 is the rate constant of phototrapping and k_{-1} is the rate constant of the decay of the trapped state. $[I^-]_t$ is the concentration of the trapped state. On illuminating the sample, $[I^-]_t$ increases with time t as

$$[I^-]_t = [I^-]_e\{1-\exp-(k_1+k_{-1})t\} \qquad (3)$$

where

$$[I^-]_e = (k_1/k_1+k_{-1})\{[I]+[I^-]_t\}$$

On turning off the light, $[I^-]_t$ simply decays as

$$[I^-]_t = [1^-]_e \exp-k_{-1}t \qquad (4)$$

The measured kinetic rate constant, k_M, which is determined from the measured change in $[I^-]_t$ with time

TABLE I

The change of absorbance and the rate constants for the two resolved components of the phototrapping kinetic curves

ΔA_A, k_A and k_A^{-1} are the change in absorbance, the rise rate constant and the decay rate constant respectively of component A. ΔA_B^C and k_B^C are the corrected change in absorbance and the corrected rise rate constant respectively of component B. k_B^{-1} is the decay rate constant of component B.

Wavelength (nm)	T(K)	Component A			Component B			Data from Fig.	$\frac{k_A}{k_B^C}$
		ΔA_A	k_A (s^{-1})	k_A^{-1} (s^{-1})	ΔA_B^C	k_B^C (s^{-1})	k_B^{-1} (s^{-1})		
530	217	0.042	$6.7 \cdot 10^{-1}$	$4.6 \cdot 10^{-4}$	0.072	$2.4 \cdot 10^{-3}$	$1.35 \cdot 10^{-2}$	4	279.2
540	217	0.094	$6.7 \cdot 10^{-1}$	$4.6 \cdot 10^{-4}$	0.030	$2.6 \cdot 10^{-3}$	$1.39 \cdot 10^{-2}$	4	257.7
645	217	0.080	$6.7 \cdot 10^{-1}$	$4.7 \cdot 10^{-4}$	0.079	$2.3 \cdot 10^{-3}$	$1.31 \cdot 10^{-2}$	6	291.3
552	217	0.062	$6.7 \cdot 10^{-1}$	$4.9 \cdot 10^{-4}$	0.063	$2.2 \cdot 10^{-3}$	$1.36 \cdot 10^{-2}$	7	304.5
EPR signal:	219	26.9 [a]	3.57	$4.6 \cdot 10^{-4}$	20.7 [a]	$1.5 \cdot 10^{-2}$	$1.36 \cdot 10^{-2}$	6	238

[a] Change of EPR signal amplitude (arbitrary units).

of illumination is not the true phototrapping rate constant k_1 but is related to it by the equation

$$k_M = k_1 + k_{-1} \tag{5}$$

The maximal measured change in absorbance ΔA_M, which is the absorbance of $[I^-]_e$, is related to the true maximal absorbance change ΔA by the equation

$$\Delta A_M = (k_1/k_1 + k_{-1})\Delta A \tag{6}$$

The true phototrapping rate constant k_B^C for Φ_B^- and the absorbance change, ΔA_B^C, corrected for the decay rate constant are listed in Table I. At 645 and 552 nm, ΔA_A is nearly equal to ΔA_B^C. ΔA_B^C is 1.71-times ΔA_A at 530 nm and 0.32-times at 540 nm. These are reasonable values considering that both BPh molecules have approximately the same extinction coefficients and that the Q_X band of Φ_B is blue-shifted with respect to that of Φ_A. However, the amplitude of the EPR signal of Φ_B^- is 0.77 that of Φ_A^-. This indicates that the two BPhs may not be perfectly symmetric.

Comparing the average phototrapping rate constant k_A and k_B^C, obtained from EPR and from optical measurements, the phototrapping of Φ_A^- proceeds 274-times faster than that of Φ_B^- (Table I).

Trapping of cytochrome c-552$^+$ and possible recombination reaction between BPh$^-$ and cytochrome c-552$^+$

At room temperature, the phototrapping of Φ_A^- is not accompanied by light-induced 552–555 nm absorbance changes due to cytochrome *c*. We assume this is due to a fast reduction by dithionite of the photochemically oxidized cytochrome. A 265 K, however, a cytochrome *c* absorbance change was observed at 552 nm (Fig. 1). The kinetics of this absorbance change are biphasic (Fig. 7, left) and do not correspond to the kinetics of the ΔA_{645} of Φ_A^- shown in Fig. 3. This kinetic behavior is unexplained at this moment. At 217 K (Fig. 7, right), the rise and fall kinetics of ΔA_{552} matches those of ΔA_{645} of Φ_A^- and Φ_B^- shown in Fig. 6. The similarity in the phototrapping kinetics of oxidized cytochrome and reduced BPh is expected if a cytochrome *c* cation is trapped for every BPh anion. However, the similarity in the decay rate constant suggests that the phototrapped Φ_A^- and Φ_B^- undergo recombination reactions with the trapped oxidized cytochrome *c*.

Discussion

Our data indicate that both Φ_A^- and Φ_B^- can be phototrapped in the photoreaction center of *Ectothiorhodospira* sp. Since the reduction of both BPh molecules is due to light absorbed only by P, the reduction is not initiated by some spurious photochemical reaction. At temperatures above 250 K, only Φ_A^- was phototrapped. Phototrapping of Φ_B^- was not observed, probably due to Φ_B^- decaying much faster than its rate of phototrapping. However, decreasing the temperature probably slows down its rate of decay more than its rate of phototrapping to such an extent that phototrapping of Φ_B^- can be observed. At 217 K, for instance, the ratio of its decay/phototrapping rate constant is about 6. Although the decay rate constant for trapped Φ_A^- also increases with temperature, its phototrapping rate constant is large enough to allow the trapping of Φ_A^- even at room temperature.

At 219 K, Φ_A^- was found to be phototrapped 274-times faster than Φ_B^-. The rate of phototrapping depends on the rate of photon capture times the quantum yield for the reduction of BPh times the trapping probability of this BPh^-. The latter probability depends on the competition between the *c*-type cytochrome and BPh^- for the reduction of P^+. The difference in the rate of phototrapping of Φ_A^- and Φ_B^- then depends mainly on the relative quantum efficiency of the reduction of Φ_A and Φ_B by P and on the relative rates of charge

recombination between P^+ and Φ_A or Φ_B. All other rates involved in the phototrapping process at the same. Since we have no measurements on the rate of charge recombination between P^+ and Φ_A^- or Φ_B^-, the measured relative rate of phototrapping of Φ_A^- and Φ_B^- cannot uniquely determine the relative quantum efficiency of the reduction of Φ_A and Φ_B by P.

The very different rate of phototrapping for Φ_A^- and Φ_B^- is difficult to understand from the crystallographic structure of the photoreaction center protein. Since the two BPhs are arranged symmetrically about the primary electron donor, one would predict symmetry also in the electron transfer processes. Direct electron transfer between P and Φ_A probably can be ruled out because the distance between them is too large to account for the rapidity of the electron transfer process (time constant of 2.8 ps at room temperature [5]). The electron transfer rate probably depends on some asymmetric bridging mechanism [19,20]. Direct electron transfer between P and Φ_B, however, is possible if the transfer rate is slower than that between P and Φ_A. Plato et al. [8], using molecular orbital calculations on the basis of the superexchange model [20] have calculated that the rate constant of electron transfer from P to Φ_A is 34-times that from P to Φ_B. These calculations take into account the structural asymmetry of the two electron transfer pathway and the influence of polar residues. If, using the same molecular orbital calculations, the recombination rates between P^+ and Φ_A^- and Φ_B^- can be calculated, the theoretical ratio of phototrapping of Φ_A^- and Φ_B^- can be determined. Since there is a paucity of experimental data on the asymmetric rate of electron transfer, a comparison with our measured ratio of 274 between the phototrapping of Φ_A^- and Φ_B^- would be a good check of such theoretical calculations.

Our data also show the reversible oxidation of the high-potential cytochrome *c*-552 (Fig. 7). This phenomenon has so far been observed only in photoreaction centers of *Rp. gelatinosa* [21]. The resemblance of the biphasic oxidation and reduction kinetics of the cytochrome to the corresponding kinetics of the two BPhs suggests that both Φ_A^- and Φ_B^- can be oxidized by the corresponding phototrapped cytochromes. The faster decay of Φ_B^- compared to Φ_A^- may be due to the faster rate of electron transfer from Φ_B^- to the *c*-hemes.

Acknowledgements

This work was supported by a grant from the Natural Science and Engineering Research Council of Canada.

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