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Evidence for the photoreductive trapping of doubly reduced bacteriopheophytin in the photoreaction center of *Ectothiorhodospira* sp.

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Continuous near-infrared illumination of *Ectothiorhodospira* sp. photoreaction centers in the presence of dithionite resulted in the bleaching of the entire bacteriopheophytin Q_X and Q_Y absorption bands. We identified the two bacteriopheophytin molecules by the bandshift of their Q_X transitions, Φ_A being red shifted and Φ_B being blue shifted. Φ_A was the first to be bleached, as it was phototrapped in the short-lived Φ_A^- state. Further illumination led to a decreased amount of Φ_A^-, concomitant with the phototrapping of very long-lived bleached Φ_A and Φ_B species. These new species were very stable under anaerobic conditions but introduction of oxygen restored the absorption bands of bacteriopheophytin. They displayed neither the electron paramagnetic resonance signal nor the broad 645 nm absorption band characteristic of singly reduced bacteriopheophytin. They are therefore suggested to be doubly reduced. Doubly reduced Φ_B was phototrapped before doubly reduced Φ_A with an activation energy of 2.76 kcal · mol^{-1} for the former and of 8.99 kcal · mol^{-1} for the latter. A large component of these activation energies being negentropic is suggested to indicate conformational changes induced by the doubly charged species.

Introduction

The primary photochemical processes in purple bacterial photoreaction center (RC) involves the transfer of an electron from the primary electron donor, P, to one of two bacteriopheophytin molecules. This molecule, Φ_A, is characterized by a red-shifted Q_X absorption band [1,2]. This very efficient reaction has a reaction rate of the order of the picosecond [3]. However, its low reduction potential and its proximity to the other four bacteriochlorin molecules [4–6] should enable excited P to undergo many other photoreduction reactions. Using the very sensitive phototrapping technique at low temperatures [7–9], we previously found [10] that P can also reduce Φ_B. We now report evidence, based on the same technique, for the double reduction of Φ_A and Φ_B. As discussed below, this double reduction is analogous to that of the primary electron acceptor, Q_A [9]. In both cases, the dianions are very stable under anaerobic conditions, can be formed only by light and not by chemical reduction. In both cases, the formation of the singly charged anion is rapid and activationless as opposed to that of the dianionic species whose activation energy is approx. 9 kcal · mol^{-1}.

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Materials and Methods

Photoreaction center was extracted from *Ectothiorhodospira* sp. as described before [11,12]. The RC was suspended in 50 mM Tris-HCl/Triton X-100 (1 : 0.01 v/v) (pH 7.4) containing 12 mg/ml sodium dithionite. Experiments at room temperature were initially carried out with the sample in an anaerobic cuvette under moderate bubbling with ultra-pure nitrogen gas. However, since the same results were obtained with the sample placed in an open cuvette with paraffin oil layered on top, all the reported experiments carried out above 0 °C were done with the latter method. For controlled temperature studies, a water-jacketed cuvette connected to a regulated temperature bath was used. For low temperature measurements, the samples were degassed by evacuation, placed in a sealed infrasil cuvette 3 mm^2 in section and cooled by N_2 gas in an Air Products Corp. Joule-Thomson cryostat.

Absorbance spectra and absorbance changes were measured with a Cary 14R spectrophotometer equipped

with a cross-illumination attachment [10]. The light was filtered by a Baird Atomic broadband interference filter centered at 930 nm. Electron paramagnetic resonance measurements were carried out with a Varian E-104A spectrometer operating at 9.01 GHz in a E321 cavity. The samples were placed in a Varian E257 variable temperature accessory.

Results

As indicated in the previous section, all the following experiments were performed in the presence of dithionite and under anaerobic conditions.

Phototrapping of Φ_A^- and reduction of Q_A^- to Q_A^{2-}

Exciting only the 880 nm band of *Ectothiorhodospira* sp. RCs at low light intensity elicited the appearance of a broad 645 nm band and the bleaching of a band at 540 nm (Fig. 1A). These changes are characteristic of the photoreduction of Φ_A to Φ_A^- [1,2,7–10]. The initial rise kinetics of the ΔA_{645} on the first light pulse lags in comparison with that produced by subsequent pulses (Fig. 1B). We attribute this delay to electron transfer from Φ_A^- to Q_A^- to form Q_A^{2-}, as was shown to occur in RC from *Rb. sphaeroides* [9]. This interpretation is supported by EPR spectroscopy: in the presence of dithionite the RC exhibited a dark EPR signal a 153 K with a g value of 2.005 and a peak to peak linewidth of

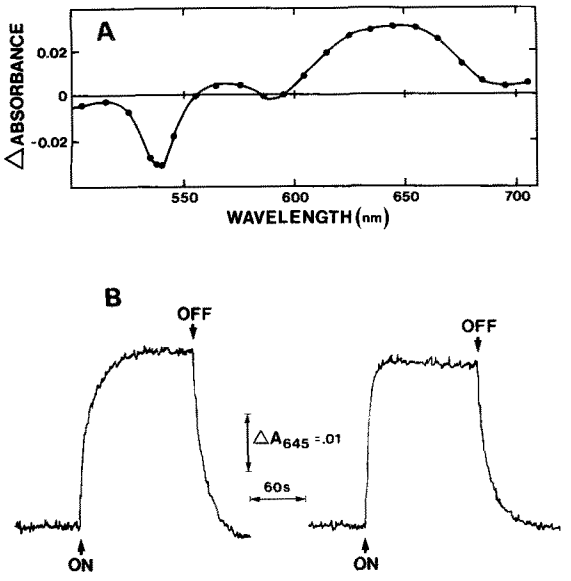

Fig. 1. (A) Difference absorption spectrum of illuminated *minus* dark samples of *Ectothiorhodospira* sp. RC preparations at room temperature. The sample was suspended in 50 mM Tris-HCl/0.1% (w/v) Triton X-100 containing 12 mg of sodium dithionite per ml of suspension. The illumination was provided by a tungsten lamp filtered with a Baird Atomic wide band interference filter centered at 930 nm. Intensity of the illumination was 1.48 ergs·cm^{-2}·s^{-1}. (B) Kinetics of the 645 nm absorbance changes of *Ectothiorhodospira* sp. RC preparations at room temperature following first (left) and second illumination (right).

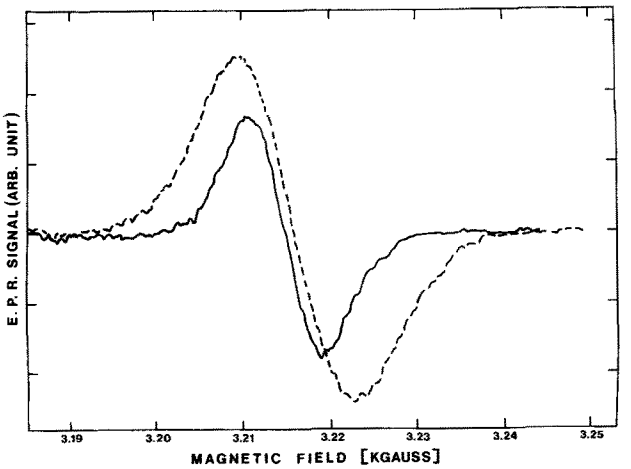

Fig. 2. EPR spectra of dithionite-treated *Ectothiorhodospira* sp. RC at 219 K. Solid line: spectrum of a sample kept in the dark. Dotted line: spectrum of a sample having received 2 min of illumination. Illumination was provided by a 500 W tungsten lamp focused through a 1 cm diameter light guide placed at an opening on the side of the sample cavity. EPR spectra were reorded at a modulation frequency of 100 kHz with microwave power of 2 mW and modulation amplitude of 3.2 G.

8.0 G characteristic of a semiquinone anion [13,14] (Fig. 2, solid line). On illumination, this signal was a g value of 2.0039 and a peak to peak linewidth of 12.8 g typical of anionic bacteriopheophytin [15] (Fig. 2, dotted line). Only the latter signal was observed after subsequent illumination.

Reduction of Φ_A^- to Φ_A^{2-}

As shown above, continuous low intensity illumination of the RCs kept at low redox potential elicits a steady-state ΔA_{645}. At high light intensities, the amplitude of the ΔA_{645} decreases with the time of illumination (Fig. 3A) at a rate directly proportional to the light intensity (not shown). A corresponding decline of the photo-induced ΔA_{535} and ΔA_{755} is also observed (Fig. 3A and B). As more clearly illustrated for the photo-induced ΔA_{755}, the kinetics has two components: one that recovers quickly upon turning off the light and one that is not recoverable as long as air is not admitted to the preparation. We will designate the first component as 'reversible' and the second one as 'stable'. As also shown by EPR (see below), the reversible component is due to the phototrapping of Φ_A into Φ_A^-. Figs. 3B and 4A show that the rise of the stable component is biphasic. The rates of increase of its slower portion and of the decrease of the reversible ΔA_{755} are the same. This indicates the disappearance of species Φ_A^- at the benefit of another stable species. This new stable species is not a photodestruction product of Φ_A since the bacteriopheophytin bands are fully restored after an exposure to air in the dark (not shown). Since only the 880 nm band was excited, this strongly suggests that the stably trapped species is reduced by the primary donor, presumably as

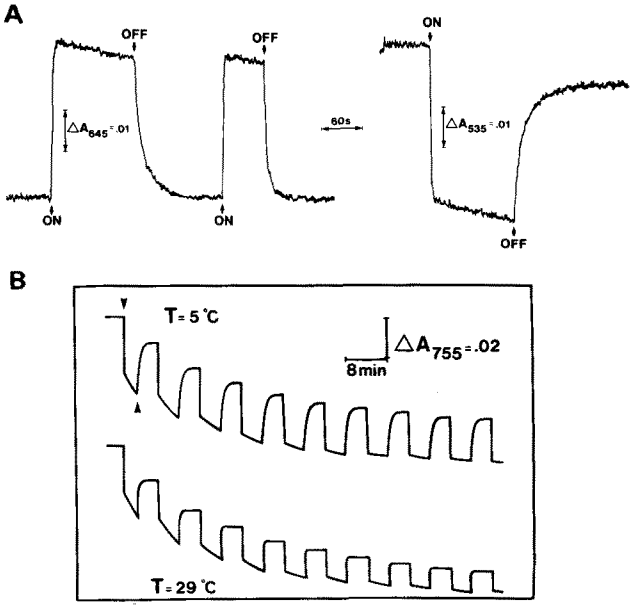

Fig. 3. (A) Reversible light-induced absorbance changes at 645 nm and 535 nm of dithionite-treated *Ectothiorhodospira* sp. RC at room temperature. Illumination intensity was 6.8 erg·cm^{-2}·s^{-1} (B) Time-course of the light-induced absorbance changes at 755 nm with intermittant illumination of dithionite-treated *Ectothiorodospira* sp. RCs at 5° C and 29° C.

Φ_A^{2-}. The faster rise kinetic component of the stable species is attributed to the phototrapping of Φ_B^{2-} (see below).

If the stable species is Φ_A^{2-} it would be expected to have no EPR signal, since, contrary to Φ_A^-, Φ_A^{2-} has no paired electrons. As shown in Fig. 4B and C, both the photoinduced EPR signal and the ΔA_{645} of Φ_A^- decrease exponentially with the time of illumination. The two reported rates are not identical because the light intensities were unmatched in the two experimental setups. The absence of any EPR signal for the new stable species is consistent with its being Φ_A^{2-}.

Reduction of Φ_B^- to Φ_B^{2-}

We equated above the slow increase of the stably bleached bacteriopheophytin to its trapping into Φ_A^{2-}. The initial rise of the stable photo-induced ΔA_{535} and ΔA_{735} which is faster than the decrease of the reversible ΔA_{645} (Figs. 3 and 4) must therefore involve Φ_B, the only other bacteriopheopytin molecule. Fig. 5A (solid line) shows the difference spectrum of that initial component obtained by subtracting the absorption spectrum taken in the dark from that taken after 2 min of illumination. This shows a bleaching of the Q_X band at

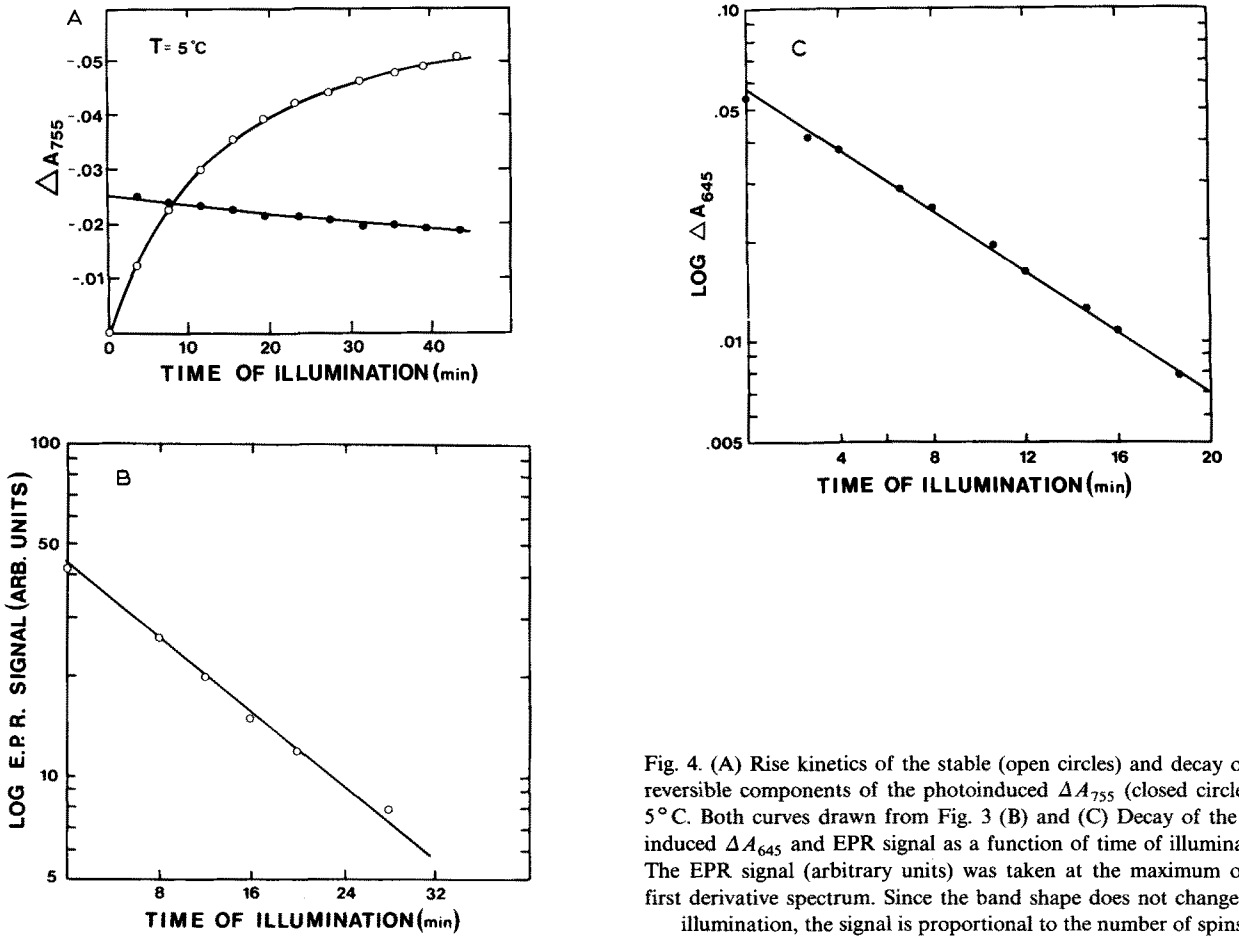

Fig. 4. (A) Rise kinetics of the stable (open circles) and decay of the reversible components of the photoinduced ΔA_{755} (closed circles) at 5° C. Both curves drawn from Fig. 3 (B) and (C) Decay of the light induced ΔA_{645} and EPR signal as a function of time of illumination. The EPR signal (arbitrary units) was taken at the maximum of the first derivative spectrum. Since the band shape does not change with illumination, the signal is proportional to the number of spins.

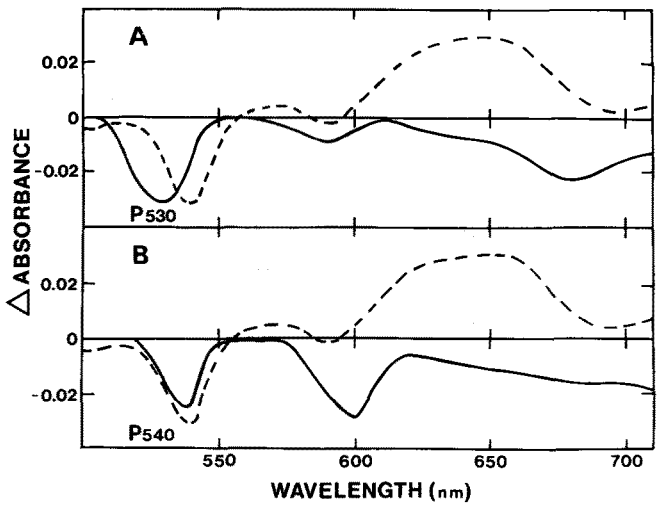

Fig. 5. Difference spectra of the two kinetic components of the stable light-induced absorbance changes in dithionite-treated RC of *Ectothiorhodospira* sp. at room temperature. (A) The difference spectrum of the fast component (solid line) was obtained by subtracting the absorption spectrum taken in the dark from that taken after 2 min of illumination. (B) The difference spectrum of the slow component (solid line) was obtained by subtracting the absorption spectrum taken after 8 min of illumination from that taken after 12 more min of illumination. Intensity of illumination was as in Fig. 3. The dotted curve, shown here for comparison purposes, is the difference spectrum of the reversible light-induced absorbance changes as shown in Fig. 1.

530 nm, which indicates that the initially phototrapped species is indeed the blue-shifted Φ_B. The absence of any 645 nm band indicates that it is not Φ_B^- but most probably Φ_B^{2-}.

Since this species is phototrapped at a faster rate than the putative Φ_A^{2-}, one would expect that a longer illumination period would reduce most of Φ_B, leaving mainly Φ_A. This deduction is borne out by the difference spectrum, taken between 8 and 12 min of illumination. This shows a bleached Q_X band at 540 nm with no positive 645 nm band (Fig. 5B, solid line), a conformation that the species phototrapped after Φ_B^{2-} is indeed the red-shifted Φ_A^{2-}.

Unlike Φ_A^-, Φ_B^- is not observed at room temperature. But at 217 K it could be monitored both by optical and EPR spectroscopy [10]. If Φ_B^{2-} is indeed phototrapped, then Φ_B^- should not be observed at 217 K. To verify this, Φ_B^- was phototrapped into Φ_B^{2-} by illuminating the RC at room temperature until half of the 755 nm band was stably bleached. The temperature was then lowered to 217 K and the the sample received light pulses. The photoinduced ΔA_{530} and ΔA_{540} (Fig. 6) were devoid of the slow rising Φ_B^- kinetics [10]. Further, ΔA_{530} is smaller than ΔA_{540}, indicating that the observed changes are due only to Φ_A^-. The disappearance of the Φ_B^- is consistent with the latter's being further reduced by P to doubly reduced Φ_B^{2-}.

Activation energy

To assess the phototrapping activation energies of

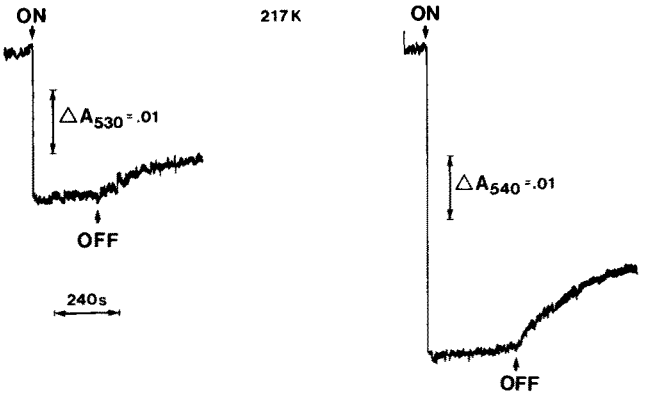

Fig. 6. Kinetics of the light-induced absorbance changes at 530 nm and 540 nm of *Ectothiorhodospira* sp. RCs at 217 K. The sample was suspended in a solution of glycerol (1:1, v/v) and 100 mM Tris-HCl/0.1% Triton X-100 containing 12 mg of sodium dithionite per ml of suspension. The sample was illuminated at room temperature until half of the 755 nm absorbance band was irreversibly bleached, the temperature was then lowered to 217 K and the kinetic curves taken.

Φ_A^{2-} and Φ_B^{2-} we resolved graphically (not shown) the rise times of the fast and slow portions of the stable photoinduced ΔA_{755} measured at different temperatures. Fig. 3 shows the rise kinetics of the ΔA_{735} at 5 °C and 29 °C. The two activation energies were obtained from a plot of the logarithm of the rate constants against the reciprocal of the temperature (Fig. 7). The activation energies for the phototrapping of Φ_B^{2-} and Φ_A^{2-} are 2.76 and 8.99 kcal · mol^{-1}, respectively. Using simple transition state theory [16], the entropy of activation for the phototrapping of Φ_B^{2-} and Φ_A^{2-} were calculated to be −53.1 and −36.4 cal · mol^{-1} · deg^{-1},

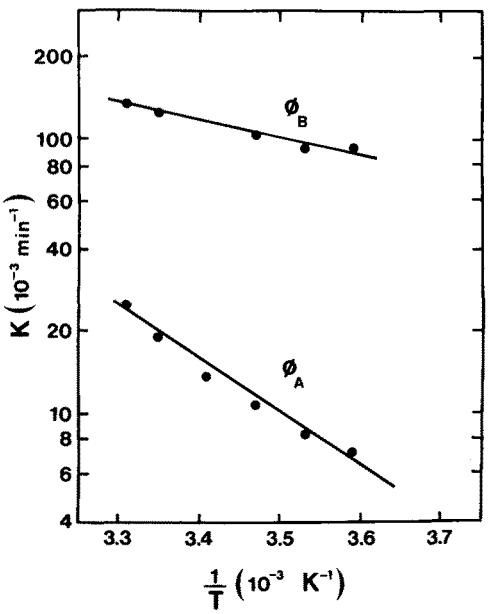

Fig. 7. Temperature dependence of K, the rate constant for the phototrapping of the Φ_B^{2-} (upper curve) and of the Φ_A^{2-} (lower curve). The rate constants were determined from the ΔA_{755} kinetics curves with repetitive illumination (Fig. 3) by resolving the curves into two components (see text).

respectively. The large entropy of activation indicates a possible structural change accompanying the electron transfer. Consistent with this hypothesis, the stable absorbance changes occur only above ice temperature. Below 273 K, only the reversible absorbance changes of Φ_A^- are found.

Discussion

The Φ_A^{2-} dianion

Illumination of dithionite-treated RCs at room temperature leads first to the trapping of the doubly reduced quinone, then to the singly reduced Φ_A^-. Sustained illumination following the trapping of Φ_A^- results in further reduction of Φ_A^-. The absence of an EPR signal and of the 645 nm absorption band is consistent with the new trapped state being Φ_A^{2-}. This is analogous to reduction of the singly reduced quinone which loses its EPR signal when it becomes doubly reduced [9]. Although the double reduction of bacteriopheophytin in vivo has not been reported, multi-electronic reduction of the porphyrin ring has been reported in the electroreduction of a number of porphyrins in dimethylforamide [17].

Φ_A^{2-} is extremely stable as long as the RC remains in an anaerobic atmosphere and is not denatured. When oxygen is introduced, Φ_A^{2-} reverts slowly back to the Φ_A state. As is also the case for the doubly reduced quinone [9], we do not know why Φ_A^{2-} is so stable while Φ_A^- has a lifetime of only a few seconds. It could be doubly protonated to form the stable $\Phi_A H_2$ or, possibly, its two negative charges may alter the immediate environment of the protein and be partially neutralized by positively charged amino acid residues.

While electron transfer from P to Φ_A is efficient, fast, activationless and occurs even at very low temperatures [18], electron transfer to Φ_A^- is inefficient, requires a large activation energy and does not occur below ice temperature. Similarly, the rate of electron transfer from Φ_A^- to Q_A is activationless and of the order of 100 ps, but it is temperature-dependent and of the order of 5 s for the transfer from Φ_A^- to Q_A^- [9]. The entropy of activation of -36.4 cal $\cdot$ mol^{-1} $\cdot$ deg$^-$ for the acceptance of two electrons by Φ_A may be due to large conformational changes of the reactants required to accommodate a doubly reduced species. The rigidity of the medium below ice temperature would prevent these changes and thus also the trapping of Φ_A^{2-}.

The Φ_B^{2-} dianion

The phototrapping of Φ_B^{2-} precedes that of Φ_A^{2-} and requires a lower activation energy but a more negative entopy of activation. Since Φ_A and Φ_B are situated almost symmetrically about P [4–6], this asymmetric electron transfer is probably related to a different protein environment. The earlier trapping of Φ_B^{2-} may be associated with a greater conformational flexibility of the B side. This hypothesis is supported by the easier extraction of B_B, the bacteriochlorophyll monomer on the B side [19] and of the carotenoid which resides on the B side.

The doubly reduced bacteriopheophytins are not intermediates of the photosynthetic electron transfer process. They are byproducts of photoreactions involving the strongly reducing primary electron donor and occur only when the normal pathway has been blocked by the reduction of Φ_A. These secondary reactions are inefficient, requiring high activation energies, and most likely do not occur in normal photosynthesis. This leaves open the question of why the primary photosynthetic reaction is so much faster and efficient.

Acknowledgement

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