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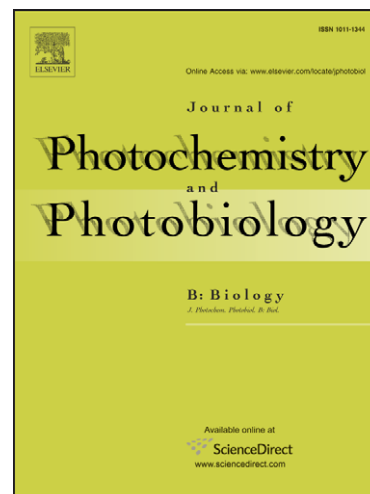
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Formation and geminate quenching of singlet oxygen in purple bacterial reaction center

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Abbreviations: $B_{A,B}$, accessory bacteriochlorophylls on *A*- and *B*-branches; (B)Chl, (bacterio)chlorophyll; Car, Carotenoid; $\Delta A_s(\lambda;t)$, time-resolved flash-induced changes in absorbance under conditions specified by the subscript (neutral when $s = n$, reducing when $s = r$) conditions; Φ^{\ddagger} , triplet quantum yield; Φ^+ , quantum yield for charge separation; $H_{A,B}$, accessory bacteriopheophytins on *A*- and *B*-branches; LDAO, lauryldimethylamine oxide; **M**, water-soluble molecule; \mathbf{M}^{\ddagger} , triplet excited state of **M**; **P**, ground state of the special pair of purple bacterial RC containing BChl *a* (P_{870}); \mathbf{P}^{\ddagger} , triplet excited state of **P**; \mathbf{P}^+ oxidized state of **P**; Q_A , primary quinone acceptor; Q_B , secondary quinone acceptor; *Rb.*, *Rhodobacter*; RC, reaction center; R_{DA} , average donor-acceptor distance; SDS, sodium dodecyl sulfate; TmS, time-resolved triplet-minus-singlet; **X**, ground-state dioxygen molecule; $^1\mathbf{X}^*$, singlet oxygen; $\zeta_S^{(1)}$ and $\zeta_S^{(2)}$, the decay times of $^1\mathbf{X}^*$ (in H_2O and D_2O , respectively) sensitized by \mathbf{P}^{\ddagger} (when $S = \text{P}$) or by \mathbf{M}^{\ddagger} (when $S = \text{M}$).

Abstract

The phosphorescence of singlet oxygen ($^1\mathbf{X}^*$) photosensitized by the carotenoidless reaction center (RC) of *Rhodobacter sphaeroides* R26.1 has been investigated, using H_2O and D_2O as the suspending media. To enhance (under neutral conditions) the triplet quantum yield of the special pair P_{870} (\mathbf{P}) by the radical pair mechanism, the quinone acceptor Q_A was removed by means of a chemical treatment. The phosphorescence signal fits the functional form $P_0 [\exp(-t/\tau) - \exp(-t/\zeta)]$, regardless of whether $^1\mathbf{X}^*$ is sensitized by \mathbf{P}^\dagger or \mathbf{M}^\dagger (where the dagger denotes triplet excitation and \mathbf{M} is a water-soluble molecule). The time constant ζ was identified with the decay time of $^1\mathbf{X}^*$; when \mathbf{P}^\dagger is the sensitizer, one finds $\zeta_{\text{P}}^{(1)} = 3.3 \pm 0.3 \mu\text{s}$, and $\zeta_{\text{P}}^{(2)} = 34 \pm 3 \mu\text{s}$, where the superscripts 1 and 2 refer to H_2O and D_2O , respectively; the corresponding values for sensitization by \mathbf{M}^\dagger (in the absence of RC) are $\zeta_{\text{M}}^{(1)} = 3.7 \pm 0.4 \mu\text{s}$, and $\zeta_{\text{M}}^{(2)} = 75 \pm 5 \mu\text{s}$. The addition of RC's to the solution of \mathbf{M} in D_2O reveals that the RC is a quencher of $^1\mathbf{X}^*$; however, for equal concentrations of the RC, $\zeta_{\text{P}}^{(2)} < \zeta_{\text{M}}^{(2)}$, showing that $^1\mathbf{X}^*$ is deactivated, after its entry into the suspending medium, mainly by the solvent or the same RC which acts as the sensitizer. The values of τ_{p} are similar in both solvents, *ca.* $2 \mu\text{s}$, but this time constant does not figure in the disappearance of \mathbf{P}^\dagger , which follows a bi-exponential course, $\alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$. The time constants τ_1 and τ_2 ($72 \pm 5 \mu\text{s}$ and $12 \pm 1 \mu\text{s}$, respectively) as well as the factor α_2 are insensitive to

the oxygen content, and quenching of \mathbf{P}^\dagger is manifested only through a three-fold reduction in the magnitude of α_1 ; these data imply the absence of dynamic quenching and heterogeneity of the RC. The mean lifetime of ${}^1\mathbf{X}^*$ inside the protein matrix is identified with τ_p , and the absence of a prompt component in the phosphorescence signal rationalized by proposing that the radiative decay of ${}^1\mathbf{X}^*$ within the RC is much slower than that in an aqueous environment.

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1. Introduction

A feature common to the reaction center (RC) of purple bacteria and that of photosystem II of plants [1,2] is the mechanism for the formation of \mathbf{P}^\dagger , the triplet state of the primary electron donor \mathbf{P} . The triplet is formed only when electron transfer to the secondary acceptors (quinones) cannot occur, either because the quinones are already reduced or altogether absent; under these circumstances, charge recombination of the initially formed ion pair gives rise to \mathbf{P}^\dagger [3–5]. Once formed, \mathbf{P}^\dagger may suffer unimolecular deactivation, or it may undergo bimolecular quenching involving electron exchange with an adjacent carotenoid (Car) [6–8], or a ground-state oxygen molecule $^3\text{O}_2(^3\Sigma_g^-)$, hereafter abbreviated as \mathbf{X} [9–12]. At this stage, the dissimilarities between the two RC's become evident. For our purpose, it is sufficient to focus on a single facet, namely the formation of singlet oxygen $^1\text{O}_2(^1\Delta_g)$, hereafter abbreviated as $^1\mathbf{X}^*$: upon illumination under aerobic conditions, RC's of photosystem II do, but those of wild purple bacteria do not, generate $^1\mathbf{X}^*$. Evidently the separation between Car and \mathbf{P}^\dagger in the RC of photosystem II is too large to conduce to an exchange coupling [2], which requires some orbital overlap; quenching of \mathbf{P}^\dagger under aerobic condition is therefore mainly a result of energy transfer to \mathbf{X} . Since the Car in the RC of photosystem II plays no role in the quenching of \mathbf{P}^\dagger , it makes more sense to compare this system with the RC of carotenoidless mutants of purple bacteria. When quinone-depleted RC's of such mutants were investigated [13], little or no evidence

could be seen for the quenching of P^{\dagger} by X . An investigation of the phosphorescence of P^{\dagger} showed that, since the energy of P^{\dagger} was in fact lower than that of ${}^1X^*$, no sensitization of ${}^1X^*$ by P^{\dagger} was to be expected [14].

The first hint that the foregoing account may be incomplete came when Liu et al. [15] reported observing the phosphorescence emission of ${}^1X^*$ from the RC of a carotenoidless mutant of the purple bacterium *Rhodobacter (Rb.) sphaeroides*. Since their main emphasis was on the influence of an externally applied magnetic field on the yield of ${}^1X^*$, a detailed kinetic investigation did not fall within the purview of their study. The present study was undertaken in the belief that it is important to confirm their observation, and even more important to take a closer look at the growth and decay of the phosphorescence signal, and compare it with the rate of decay of P^{\dagger} under anaerobic and aerobic conditions. Using a sensitive detection system with appropriate time resolution, we have found results that shed, as will be shown below, much new light on the generation and deactivation of ${}^1X^*$ in the purple bacterial RC.

2. Material and Methods

2.1 Purification of chemically Q_A -depleted RC's of *Rb. sphaeroides* R26.1.

Cells of *Rb. sphaeroides* R26.1 were grown under anaerobic conditions at room temperature as described by Cohen-Bazire et al. [16]. The purification of control RC's containing Q_A was carried out as described previously [17]. To enhance the yield of

the triplet excited state of **P** in RC's of *Rb. sphaeroides* R26.1 under neutral conditions, the control RC preparation was subjected to a chemical treatment developed by Okamura et al. [18]. The conditions chosen to remove Q_A from the RC's included a dilution of the RC sample to a final optical density of 0.2 cm⁻¹ at 800 nm, and a treatment with 4% (vol/vol) lauryldimethylamine oxide (LDAO) and 10 mM *o*-phenanthroline in 20 mM Tris-HCl pH 8.0 for 6 h at 25 °C. After incubation, the RC's were loaded onto a Whatman DE52 anion exchange column (Bodman Industries, Aston, PA) equilibrated with 0.1% (vol/vol) LDAO, 20 mM Tris-HCl pH 8.0 (Buffer A). After an extensive wash with Buffer A to remove free pigments, the RC's were eluted from the column with 1 M NaCl Buffer A. The pooled RC's were diluted ten-fold in Buffer A and further purified on a HiTrap Q anion exchange column (Amersham Pharmacia Biotech., Buckinghamshire, England). The RC's were eluted with a linear gradient of increasing NaCl concentration from 0–0.5 M. RC fractions with an absorbance ratio $A_{280}/A_{800} = 1.35 \pm 0.05$ were pooled and concentrated by using centricon YM 100 centrifugal filter units in a Kubota centrifuge model 6900 (Kubota Corp., Tokyo, Japan) at 4000g. The concentrated RC samples were passed through a Superdex 200 size exclusion column (Amersham Pharmacia Biotech., Buckinghamshire, England) to ensure the RC samples did not contain free pigments. The RC samples were pooled and kept at –20 °C until use. The polypeptide pattern of RC was analysed by SDS-PAGE as described by Laemmli [19] using a total acrylamide

content of 15%. The SDS polyacrylamide gel was stained with Coomassie R 250 (Figure 1, inset).

2.2 Absorption spectroscopy.

Absorption spectra were recorded by using a commercial Shimadzu spectrophotometer Model UV-1601PC (Shimadzu Scientific Instruments, Inc. Columbia, MD).

2.3 Transient Absorption spectroscopy.

Photo-induced changes in the absorbance of the sample under investigation will henceforth be denoted as $\Delta A(\lambda; t)$, where λ is the wavelength of the monitoring light and t stands for the delay between photoexcitation of the sample and the instant at which the resulting change in absorbance is probed. For a given delay, a plot of $\Delta A(\lambda; t)$ against λ will be called a difference spectrum; for a given λ , we will use the symbol $\Delta_w(t)$, with $w = \lambda/\text{nm}$, and will refer to a plot of $\Delta_w(t)$ against t as a kinetic trace. The difference spectra presented here were recorded by using a home-built multi-channel kinetic spectrometer whose components and operation have been described in two recent publications [17,20]. The principal components of the apparatus used for recording the kinetic traces have also been described before [21];

the monitoring source was replaced by a pulsed xenon arc, but all other details remain unchanged. Though the overall time resolution of the detector electronics is a few ns, the dead time of the apparatus (due to blinding of the photomultiplier tube by the scattered laser radiation) is 200–300 ns (depending on the monitoring wavelength). In what follows, the phrase “initial absorbance” will refer to the value at the end of the dead time. Anaerobic conditions were created by directing a stream of argon to the surface of the RC sample inside the cuvette before and during the measurements. Under aerobic conditions, molecular oxygen was left to diffuse freely from atmosphere to the RC sample; the sample was replaced after 128 exposures to the excitation light, even though no degradation of the sample could be discerned by examining its absorption spectrum. A control experiment confirmed that 1000–1500 laser shots were needed to bring about a noticeable irreversible loss of absorption around the Q_y band of **P**. All the assay buffers were 50 mM Tris-HCl pH 8.0.

2.4 Time-resolved singlet oxygen measurements.

Time-resolved phosphorescence of $^1\mathbf{X}^*$ was studied in air saturated solutions of Q_A -depleted RC samples. The excitation pulse, which had a duration of about 7 ns and an energy of 5–10 mJ was delivered by the third harmonic (355 nm) of the same Nd:YAG laser (operating at 10 Hz) as that used for recording the kinetic absorption traces; to attenuate the residual contribution of the fundamental output (at 1064 nm), a water-filled cylindrical cell (path length 30 cm) was placed in the path of the excitation

beam. Phosphorescence of $^1\text{X}^*$, viewed at a right angle to the direction of excitation beam, was focused, with the aid of a convex lens and a parabolic mirror, on the photocathode of an infrared-sensitive photomultiplier tube R5509-42 (Hamamatsu Photonics K. K., Hamamatsu, Japan). For suppressing unwanted radiation (particularly the luminescence of the sensitizer), a long pass filter (cutoff wavelength 780 nm) and a band pass interference filter (center wavelength 1273 nm, FWHM 12 nm) were placed between the collecting lens and the photomultiplier. The output of the photomultiplier, converted to a voltage by using an anode load resistor of 50 Ω , was amplified using a 350 MHz bandwidth Spectra Physics preamplifier model 70723 (Spectra-Physics Lasers Division, Mountain View, CA) and fed to 600 MHz Agilent Oscilloscope model Infinium 54830B (Agilent Technologies, Palo Alto, CA). Near-infrared phosphorescence of singlet oxygen was investigated in both H_2O and D_2O . When using D_2O , the RC samples were concentrated by using centricon YM 100 centrifugal filter units in a Kubota centrifuge model 6900 (Kubota Corp., Tokyo, Japan) at 4000g. Then, the RC samples were newly diluted by adding 1 mL of D_2O in each filter unit. The concentration-dilution cycle was repeated at least 10 times to avoid traces of H_2O in the final solution. Neither Tris nor LDAO was added to the solution during and after the exchange of H_2O by D_2O . The pD was not adjusted.

3. Results

It will be convenient to state at the outset an overall feature of the difference spectra which are to be presented below. On the basis of our previous work [17], we can represent any transient spectrum recorded in this study as a superposition of two difference spectra: $\Delta A(\lambda; t) = a^\dagger(t)\Delta A^\dagger(\lambda) + a^+(t)\Delta A^+(\lambda)$, with $a^\dagger \geq 0$, and $a^+ \geq 0$. The spectrum labeled $\Delta A^\dagger(\lambda)$ arises from the conversion of \mathbf{P} into \mathbf{P}^\dagger ; that labeled $\Delta A^+(\lambda)$, from the conversion of \mathbf{P} into \mathbf{P}^+ . Using the abbreviations $t_1 = 1 \mu\text{s}$ and $t_2 = 200 \mu\text{s}$, the change in the composition of a difference spectrum in which $a^\dagger(t_1) > 0$ can be summarized as follows: $a^\dagger(t_2) = 0$, and $a^+(t_2) = a^+(t_1) = a^+$. When the need will arise for distinguishing between neutral and reducing conditions, we will add a subscript (n or r) by writing, for example, $\Delta A_n(\lambda; t) = a_n^\dagger(t)\Delta A^\dagger(\lambda) + a_n^+(t)\Delta A^+(\lambda)$.

The results will be grouped into three parts, dealing with: characterization of the RC preparation, the kinetics of the photo-induced changes, and the detection of the phosphorescence of $^1\mathbf{X}^*$.

3.1 The RC preparation

Figure 1 shows the ground state absorption spectrum of RC from *Rb. sphaeroides* R26.1 after the chemical treatment with LDAO and *o*-phenanthroline based on the procedure described by Okamura and coworkers [18]. Notwithstanding the overall similarity of the spectrum to that of control RC's, subtle changes are identifiable in the near infrared region. In particular, the initial ground state absorption spectrum of the

chemically treated RC's exhibits a decrease in absorbance and a slight blue shift of the Q_y band of **P** (865 nm) and an increase in the absorbance of the Q_y band of accessory bacteriopheophytin *A* and *B* ($H_{A,B}$) molecules (760 nm); since such changes have been seen in iron-depleted RC's [22], we conclude that the chemically treated RC's have not only lost the quinones Q_A and Q_B , but also, to a certain extent, the non-heme iron (see below).

The chemical procedure used for the removal of quinones did not prove effective in extracting Q_A from all RC's. This was inferred by examining the time dependence of the $\Delta A_n(\lambda; t)$, the difference spectrum under anaerobic and non-reducing conditions; an example is presented in the inset of Figure 2, which shows that

$$\Delta A_n(850 \text{ nm}; t_2) = 0.5 \Delta A_n(850 \text{ nm}; t_1). \text{ Had our preparation been entirely free of}$$

quinones, we would have found $\Delta_{850}(t_2) \approx 0$. The difference

$$\Delta A_n(\lambda; t) - \Delta A_n(\lambda; t_2) = a_n^\dagger(t) \Delta A^\dagger(\lambda)$$

gives the contribution from the Q_A -depleted RC's in our heterogeneous sample of RC's under anaerobic non-reducing conditions. The relative abundance of the quinone-containing and quinone-depleted RC's will now be estimated by using two approaches, both of which rely on comparing the bleaching signals at 850 nm under neutral (n) and reducing (r) conditions.

The initial contributions made by \mathbf{P}^\dagger and \mathbf{P}^+ to the difference spectrum under neutral conditions are $I_n^\dagger(\lambda) \equiv a_n^\dagger(t_1) \Delta A^\dagger(\lambda)$ and $I_n^+(\lambda) \equiv a_n^+ \Delta A^+(\lambda)$, respectively, and $I_n^\dagger(850 \text{ nm}) = I_n^+(850 \text{ nm})$. We now follow Chidsey et al. [23] and introduce two assumptions. We suppose, in the first place, that the molar absorption coefficients of

\mathbf{P} and \mathbf{P}^\dagger do not change when the quinones are extracted or reduced; the initial contributions of \mathbf{P}^\dagger and \mathbf{P}^+ (when present at concentrations C^\dagger and C^+ , respectively) can then be expressed as $I_n^\dagger = l(\epsilon_w^\dagger - \epsilon_w^0)C^\dagger(t_1)$ and $I_n^+ = l(\epsilon_w^+ - \epsilon_w^0)C^+(t_1)$, respectively, where l is the optical path length; ϵ_w^0 , ϵ_w^\dagger , and ϵ_w^+ are the molar absorption coefficients (at a wavelength $\lambda = w$ nm) of \mathbf{P} , \mathbf{P}^\dagger and \mathbf{P}^+ , respectively. We assume next that $(\epsilon_w^\dagger - \epsilon_w^0) \approx (\epsilon_w^+ - \epsilon_w^0)$, which amounts essentially to neglecting ϵ_{850}^\dagger and ϵ_{850}^+ in comparison with ϵ_{850}^0 . We can replace C^\dagger by $f\Phi^\dagger C_0$ and C^+ by $f\Phi^+ C_2$, where C_0 and C_2 are the concentrations of the quinone-depleted and quinone-containing RC's, respectively, f is the fractional depopulation due to photoexcitation, Φ^\dagger is the efficiency of triplet formation following charge recombination in quinone-depleted RC's, and Φ^+ the quantum yield for charge separation. According to Chidsey et al. [23] $\Phi^\dagger = 0.32$ and $\Phi^+ = 1$, which leads to the conclusion that $C_0/C_2 \approx 3$.

The fraction of Q_A -depleted RC's can also be determined by using an independent argument. It follows from what has been said above that, under fully reducing conditions, the pool of Q_A -containing RC's will also participate in the radical pair mechanism and generate \mathbf{P}^\dagger ; consequently, one will now have $a_r^+ = 0$, and $\Delta A_r(\lambda; t) = a_r^\dagger(t)A^\dagger(\lambda)$, with $a_r^\dagger(t_1) > a_n^\dagger(t_1)$. The factor $a_r^\dagger(t_1)/a_n^\dagger(t_1)$, determined by adding 5 mM $\text{Na}_2\text{S}_2\text{O}_4$, came out to be close to 1.3; if we *assume* that the efficiency of triplet formation in quinone-depleted RC's is the same as that in quinone-containing RC's, we can write $a_r^\dagger(t_1)/a_n^\dagger(t_1) = C/C_0$, where C_0 is the concentration of the quinone-

depleted RC's and $C = C_0 + C_2$ the total concentration of the RC's, the relative abundances of the two pools turn out to be 75% and 25%, respectively.

3.2 Triplet kinetics

Two examples of ΔA_{850} , obtained by irradiating our RC samples under aerobic and anaerobic conditions, are shown (Figure 2). As is to be expected from the foregoing discussion of the inset in the figure, a long-lived component is found in each case.

Satisfactory fits to both traces were found by choosing the function

$$\Delta A_{850} = -[\alpha_0 + \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)],$$

and the optimum values of the fitting parameters are displayed in the table appearing as an inset in Figure 2; it is

noteworthy that the time constants τ_1 and τ_2 ($72 \pm 5 \mu\text{s}$ and $12 \pm 1 \mu\text{s}$, respectively) as

well as the pre-exponential factor α_2 are unaffected by the presence of molecular

oxygen. The fact that at a delay longer than $150 \mu\text{s}$ the two traces merge into each

other shows that the presence of molecular oxygen in solution does not perturb charge separation in those RC's where Q_A (or Q_B) are present.

The ground state absorption spectrum of the Q_A -depleted RC's suffered, after it was

exposed to a large number of laser pulses (1000–1500), a noticeable irreversible loss of

absorbance in the Q_y band of **P**, but no significant changes could be noticed in the Q_y

bands of the accessory bacteriochlorophyll A and B ($B_{A,B}$) molecules and $H_{A,B}$ (Figure

1).

3.3 Detection of the phosphorescence of $^1\mathbf{X}^*$

The observed reduction in the magnitude of α_1 under aerobic conditions leads one to expect the formation of $^1\mathbf{X}^*$ in Q_A -depleted RC samples. As a prelude, the performance of the detection apparatus was tested by recording the phosphorescence of $^1\mathbf{X}^*$ photosensitized by methylene blue in H_2O and D_2O . The oscilloscope signal, presented in Figure 3, shows the build-up and subsequent decay of the concentration of $^1\mathbf{X}^*$ (in H_2O) after the laser pulse. This, as well as the other signals representing the phosphorescence of $^1\mathbf{X}^*$ in the various systems examined here, could be well fitted to the relation $S(t) = A(\tau - \zeta)^{-1} [\exp(-t/\tau) - \exp(-t/\zeta)]$, with $A > 0$; here ζ stands for the lifetime of $^1\mathbf{X}^*$, but the physical significance of τ depends on the particular system under examination. When \mathbf{M}^\dagger is the sensitizer (where \mathbf{M} denotes a water soluble molecule and the dagger symbolizes triplet excitation), τ corresponds to the lifetime of \mathbf{M}^\dagger . The analysis of aqueous systems led to the following results:

$\tau_M^{(1)} = 2.3 \pm 0.2 \mu\text{s}$, and $\zeta_M^{(1)} = 3.7 \pm 0.4 \mu\text{s}$; $\tau_M^{(2)} = 2.2 \pm 0.2 \mu\text{s}$, and $\zeta_M^{(2)} = 75 \pm 5 \mu\text{s}$, where

the superscripts 1 and 2 refer to H_2O and D_2O , respectively, and the subscript \mathbf{M} has

been added to distinguish these values from their counterparts, to which the subscript

\mathbf{P} will be attached, found by using suspensions of the RC, in which \mathbf{P}^\dagger acts as the

sensitizer. Our value of $\zeta_M^{(1)} = 3.7 \pm 0.4 \mu\text{s}$ is in agreement with a recent determination

[24,25].

Next, emission from $^1\mathbf{X}^*$ endogenously photosensitized by Q_A -depleted RC's was monitored in D_2O after extensive removal of water in the RC sample. Figure 4 shows the normalized phosphorescence signals of three samples, two of which contained a known percentage (vol/vol) of H_2O . In the absence of water, we found $\tau_p^{(2)} = 2.2 \pm 0.2$ μs , and $\zeta_p^{(2)} = 34 \pm 2$ μs ; the latter value is in fair agreement with the result (43 μs) found by Liu and co-workers [15], who did not report the rise time of their signal. The emission signal disappeared when the content of molecular oxygen was reduced by introducing argon or adding NaN_3 to the sample. The addition of water led to a decrease in the intensity of $^1\mathbf{X}^*$ emission and a shortening of the lifetime; ζ_p was found to be 26 ± 1 μs and 20 ± 1 μs for 4% and 10% H_2O , respectively, indicating that the decaying parts of the curves in Figure 4 are determined by only the deactivation of $^1\mathbf{X}^*$. No significant changes were observed in the magnitude of τ_p upon the addition of H_2O . A small but detectable signal could be recorded from Q_A -depleted RC samples in H_2O (Figure 4, inset), and its analysis led to the following values: $\tau_p^{(1)} = 1.8 \pm 0.2$ μs , and $\zeta_p^{(1)} = 3.3 \pm 0.3$ μs .

The phosphorescence of $^1\mathbf{X}^*$ when endogenously sensitized by Q_A -depleted RC samples was compared with that recorded after exogenous sensitization and addition of RC to D_2O as a quencher (Figure 5). The data were analyzed in terms of the Stern-Volmer equation, $1/\zeta_M^{(2)}(C_Q) = 1/\zeta_M^{(2)}(0) + k_q C_Q$, where C_Q is the concentration of quencher (that is, RC), and $\zeta_M^{(2)}(0)$, the lifetime of $^1\mathbf{X}^*$ in the absence of the RC. The

bimolecular rate constant for quenching, k_q , came out to be $(1.1 \pm 0.1) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, showing that not surprisingly the bulky RC protein matrix is in fact a very efficient quencher of singlet oxygen as other bulky proteins [26]. Be that as it may, for the same concentration of the RC, $\zeta_{\text{P}}^{(2)} < \zeta_{\text{M}}^{(2)}$. The large difference between $\zeta_{\text{M}}^{(2)}$ and $\zeta_{\text{P}}^{(2)}$ implies that both the suspending medium and the RC protein matrix deactivate $^1\text{X}^*$; however, some other implications of this result will be discussed in the next section.

4. Discussion

Analysis of the data plotted in Figure 2 has led us to conclude that, if the yield of triplet formation from charge recombination does not depend on the presence or absence of the quinones, only 75% of the RC's in our preparations are quinone-depleted. Two independent approaches have been followed to determine the fraction of Q_A -depleted RC's in our preparation, and both agree well. However, the determined fraction of Q_A -depleted RC's in our preparation shows that we fall short of the result reported by Okamura et al. [18] who attained a 90% depletion of Q_A in their chemically treated RC's. It is worth mentioning here that although the Q_A extraction from RC has been followed as described in [18]; residual Q_A in the RC's were not extracted in acetone-methanol (1:1, vol/vol) when a large-scale preparative procedure was followed; this departure might have contributed to the observed difference. As is to be expected, the contribution made by this Q_A -containing fraction

to each kinetic trace in Figure 2 appears, over the time scale of our interest, as a constant background that does not affect our analysis of the kinetics of the Q_A -depleted fraction and our conclusions about singlet oxygen photogeneration and quenching in carotenoidless purple bacterial RC's (vide infra).

For discussing the contribution to Δ_{850} made by the remaining 75% RC's, it is pertinent to recall some results from two closely related previous investigations. In one study, Kirmaier et al. [27] reported that a small fraction of iron-depleted RC's produces, under neutral conditions, P^{\ddagger} (through the radical pair mechanism), and that the lifetime of these triplets is rather short (about 25 μ s); it should also be noted that, in order to remove non-heme iron, they used *o*-phenanthroline as iron chelator. Shuvalov and Parson [13] found that the lifetime of P^{\ddagger} is about 12 μ s in the presence of Q_A^- (a situation that cannot arise in our experiments) and about 90 μ s if Q_A is doubly reduced or removed (along with non-heme iron) by treating the RC's with SDS. Since the contribution to Δ_{850} made by P^{\ddagger} seems to follow, in our case, a bi-exponential behavior, with lifetimes of nearly 70 μ s and 12 μ s, it is reasonable to conclude, in the light of the results briefly recounted above and in the subtle changes observed in the near infrared absorption spectrum of our RC preparation, that we are dealing with two groups of Q_A -depleted RC's: those where P^{\ddagger} is long-lived must have lost Q_A but not non-heme iron, while those where P^{\ddagger} is short-lived must in addition be iron-depleted.

Our results also show that oxygen affects only the RC's with the longer lifetime (to be called the vulnerable variety); furthermore, the triplets (\mathbf{P}^\dagger) in this group are quenched instantaneously (that is, within the dead time of the apparatus), which can only happen if a ground state oxygen molecule \mathbf{X} is already in the vicinity of \mathbf{P}^\dagger . Quenching of this kind is known as static quenching [28,29]. Since the RC's with the shorter lifetime (in the iron-depleted group) are immune to static quenching, one must conclude that, in these RC's, \mathbf{X} is unable to get as close to \mathbf{P}^\dagger as in the vulnerable variety. The reason for such a change in vulnerability is not known, but subtle changes in the RC structure are not precluded as discussed below.

We would like to pause here and recall that \mathbf{X} can quench the lowest triplet state (T_1) of a molecule with a singlet ground state (S_0) either through energy transfer or by catalyzing, on account of its paramagnetic character, the $T_1 \rightarrow S_0$ intersystem crossing [30,31]; the former process is usually very fast, but even the latter is able to shorten the triplet lifetime of a photoprotecting xanthophylls in the light-harvesting complex LHCII from 9 μs to 2-4 μs [32]. It follows therefore that though \mathbf{P}^\dagger is reported to have a triplet energy slightly lower than the energy of $^1\mathbf{X}^*$ [14,33], the quenching of \mathbf{P}^\dagger by \mathbf{X} will become negligible only when the two species cannot form an encounter complex within the lifetime of \mathbf{P}^\dagger . The observed non-vulnerability of a pool of RC's to quenching should therefore be ascribed to a low local concentration of \mathbf{X} in the vicinity of \mathbf{P}^\dagger .

We return once more to the work of Shuvalov and Parson [13] on RC's treated with SDS (and lacking both Q_A as well as non-heme iron) and recall that although they observed mono-exponential decay of P^\dagger (with a lifetime of about 90 μ s), they did report that P^\dagger could not be quenched by X in their samples. Taken together, the data obtained by using RC's lacking non-heme iron and/or Q_A indicate that the lifetime of P^\dagger as well as vulnerability to oxygen are extremely sensitive to structural details.

To subject our data to some quantitative analysis, let us note that sensitization of $^1X^*$ by P^\dagger requires electron exchange, and that the rate constant for such a process declines precipitously with the donor-acceptor distance [34,35]. A static quenching rate constant larger than or equal to 10^7 s^{-1} would amount (on our time scale) to instantaneous quenching of a vulnerable RC. Let us now assume that R_{DA} the average donor-acceptor distance in the native RC's is 0.6 nm or larger, and allow for a slight structural modification of the chemically treated RC, amounting to an increase of 0.1 nm in R_{DA} . According to some model calculations [35], even such a small increase can lower the donor-acceptor coupling by a factor of up to nearly 100, and therefore reduce the sensitization rate constant by a factor as large as 10^4 . If we accept an upper limit of 10^9 s^{-1} for the sensitization rate constant [36], the non-occurrence of sensitization in our iron-depleted RC's, as well as in those investigated by Shuvalov and Parson [13], does become comprehensible. The assumed upper limit implies, of course, that X does not form a tight complex with P , which too is consistent with the foregoing considerations.

There still remains the problem of accounting for an observable rise time for the phosphorescence of ${}^1\mathbf{X}^*$. This can be understood if we assign the time constant τ_p to the lifetime of ${}^1\mathbf{X}^*$ within the protein environment, and assume that the radiative rate constant of ${}^1\mathbf{X}^*$ in this environment is significantly smaller than in an aqueous environment; the assumption is necessary to ensure that whatever emission is observed emanates from those molecules of ${}^1\mathbf{X}^*$ that manage to find their way into the suspending medium. The radiative lifetime of ${}^1\mathbf{X}^*$ is known to depend on the perturbations introduced by the environment [37], and in the case of the RC, the field of the iron ion might also play some role; the mean lifetime (τ_p) within the protein environment will be determined essentially by the rate constants of quenching (due to reaction with the ground state of \mathbf{P} and possibly with other constituents of the protein matrix) and exit into the suspending medium. It has been stated above that $\zeta_p^{(2)} < \zeta_M^{(2)}$; this result implies that even when ${}^1\mathbf{X}^*$ emerges from the interior of the protein to the surrounding solvent layer, it is more likely to be deactivated by the solvent or the same RC where it was sensitized (geminate quenching) than by a different RC (non-geminate quenching).

Though the energy of \mathbf{P}^\dagger is reported to be lower than that of ${}^1\mathbf{X}^*$ [14], the results announced by Liu et al. [15] and confirmed in this paper show that the energy deficit is not large enough to rule out, at room temperature and in the absence of the Car, the occurrence of the energy transfer process $\mathbf{P}^\dagger + \mathbf{X} \rightarrow \mathbf{P} + {}^1\mathbf{X}^*$. These observations

corroborate the suggestion, made more than fifty years ago by Griffiths and coauthors [38], that the presence of the Car is crucial for the survival of purple bacteria.

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Figure captions

Figure 1. Set of absorption spectra of control (gray solid line) and Q_A -depleted (dark lines) RC's of *Rb. sphaeroides* R26.1 (normalized at 802 nm) showing bleaching caused by exposure to a large number (1000–1500) of laser flashes under aerobic conditions (non-exposed, dark solid line; intermediate exposure, dark dotted line and extensive exposure, dark dashed line). Absorption spectra of control and Q_A -depleted RC's were recorded in 0.1% LDAO, 20 mM Tris-HCl pH 8.0 and D_2O , respectively. Inset, SDS-PAGE analyses of the polypeptide pattern of control RC.

Figure 2. Kinetic behavior of the change in absorbance at 850 nm of Q_A -depleted RC's of *Rb. sphaeroides* R26.1 under anaerobic (black) and aerobic (grey) conditions. Each curve (an average of 768 traces) was fitted to

$$\Delta A_{850} = -[\alpha_0 + \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)];$$

also shown are the resulting values of τ_1 , τ_2 , $a_i \equiv 10^3 \times \alpha_i$ and the corresponding residuals. Inset: Flash-induced difference spectra under anaerobic conditions at 1 μ s (black curve) and 200 μ s (dotted curve); each spectrum is an average of 64 records. RC concentration is 3.5 μ M.

Figure 3. Temporal profile (obtained by averaging 2048 traces) of singlet oxygen phosphorescence at 1270 nm by methylene blue in water.

Figure 4. Normalized temporal profile (obtained by averaging 1024 traces) of singlet oxygen phosphorescence at 1270 nm in Q_A -depleted RC's of *Rb. sphaeroides* R26.1 dispersed in D_2O (black trace), 4% H_2O (dark gray trace), 10% H_2O (light gray trace) and H_2O (inset). RC concentration is $3.5 \mu M$ in all the assays.

Figure 5. Stern-Volmer analysis of the deactivation of singlet oxygen photosensitized by methylene blue when using RC as a quencher.

ACCEPTED MANUSCRIPT

