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ORIGINAL PAPER

Singlet oxygen generation in the reaction centers of *Rhodobacter sphaeroides*

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Abstract Singlet oxygen ($^1O_2$) generation in the reaction centers (RCs) of *Rhodobacter sphaeroides* wild type was characterized by luminescent emission in the near infrared region (time resolved transients and emission spectra) and quantified to have quantum yield of 0.03 ± 0.005. $^1O_2$ emission was measured as a function of temperature, ascorbate, urea and potassium ferricyanide concentrations and as a function of incubation time in H_2O:D_2O mixtures. $^1O_2$ was shown to be affected by the RC dynamics and to originate from the reaction of molecular oxygen with two sources of triplets: photoactive dimer formed by singlet-triplet mixing and bacteriopheophytin formed by direct photoexcitation and intersystem crossing.

Keywords Photosynthetic bacteria · Purple bacteria · Photoinhibition · Triplets · Quantum yield · ROS

Introduction

In photosynthetic organisms light overloading conditions generally lead to photoinhibition of photosynthesis and photodestruction of photosynthetic apparatus (Knox and Dodge 1985). Both processes have been related with the generation of singlet oxygen ($^1O_2$). Consequently, $^1O_2$ production can represent low crop yields and several strategies to decrease production of $^1O_2$ or to decrease its damaging properties are under investigation (Knox and Dodge 1985; Hideg et al. 2000, 2006, 2007).

Formation of $^1O_2$ in Photosystem II (PSII) of plants was invariably confirmed by the detection of its characteristic Near-Infrared (NIR) emission at 1,270 nm (Macpherson et al. 1993; Telfer et al. 1994). The generation mechanism involves reduction of quinone acceptors, favoring back-electron transfer between reduced pheophytin and oxidized P680, which leads to the formation of mainly P680 triplets (Vass et al. 1992). However, the details of $^1O_2$ generation are still a matter of debate and triplets derived from other RC pigments have also been detected (Durrant et al. 1990). By using chemical-trapping experiments, Rinalducci and co-workers have shown the formation of $^1O_2$ in trimer antenna-complex proteins and suggested the direct generation of $^1O_2$ by oxygen quenching of triplet chlorophyll species formed after light absorption and intersystem crossing (ISC) (Rinalducci et al. 2004). The role of Type II photosensitization reaction with chlorophyll triplets generated by photoexcitation and ISC has also been mentioned in a recent review (Krieger-Liszkay 2004).

In spite of the homology between PSII and RCs of purple bacteria, the photosynthetic apparatus of wild-type purple bacteria is less sensitive to photoinhibition. Photoactive pigment (P) triplets ($^3P^*$) have been shown to occur in the RCs of purple bacteria (Laible et al. 2003). Carotenoidless strains are highly sensitive to photodamage and generation of $^1O_2$ has been detected by chemical trapping methods (Tandori et al. 2001) and by direct NIR measurements (Liu et al. 2005; Arellano et al. 2007). The absence of any detectable amount of $^1O_2$ in RCs of wild-type purple bacteria has been related with efficient carotenoid (Car) quenching of triplets (Tandori et al. 2001; Liu et al. 2005; Arellano et al. 2007). In a recent work,

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Donohue et al. have shown that *Rhodobacter sphaeroides* (*Rb. sphaeroides*) has specific gene expression trends to protect itself from $^1O_2$ generated internally or externally by type II photosensitization with methylene blue (MB) (Anthony et al. 2005).

In the RCs of wild-type strains, two sets of pigment cofactors are arranged in two branches (labeled A and B): photoactive dimer, two monomeric bacteriochlorophylls (BChls), two molecules bacteriopheophytin (BPh) and two quinones. Also a molecule of Car is localized near $BChl_B$ in the RC structure of wild-type bacteria (Deisenhofer and Michel 1989). After formation of the electronically excited singlet state $^1P^*$ a rapid charge separation takes place along the A branch. Electron transfer follows a sequential mechanism where the anion radicals of $BChl_A^-$, BPh_A^- and Q_A^- are sequentially formed with time constants of 3, 0.9 and 200 ps, respectively, before reaching the secondary acceptor (Q_B) (Kirmaier et al. 1985, 2005; Holzapfel et al. 1989; Holzwarth and Muller 1996).

In this work, we have investigated the generation of $^1O_2$ from RC obtained from *Rb. sphaeroides* wild type containing both quinone acceptors (Q_A and Q_B) under pulsed light illumination (10 mJ/pulse, 1–10 Hz). Reversible one-electron transfer between P and quinone acceptors was detected as a result of light activation. Both, slow and fast dark recovery of P^+ from Q_B^- ($t_{1/2} = \sim 1$ s) and from Q_A^- ($t_{1/2} \sim 100$ ms), respectively, were observed, indicating charge recombination via the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ equilibrium (Knox et al. 2005). Under these circumstances, the detection of $^1O_2$ had been tried by spin trapping with TEMP without success, suggesting again the absence of $^1O_2$ generation. However, in order to gain better knowledge in the generation of $^1O_2$, we have decided to study this process using a more sensitive and selective method (Gabrielli et al. 2004; Severino et al. 2003; Niedre et al. 2006; Jarvi et al. 2002). We report the proof that $^1O_2$ is generated in the RCs of wild-type *Rb. sphaeroides*, the quantum yield of this process and the main sources of triplet species.

Experimental

Materials

All solvents used were of spectroscopic grade. Water was bi-distilled from all glass apparatus and was further purified via a millipore Milli-Q system. Urea was recrystallized from methanol. Ortho-phenanthroline and potassium ferricyanide (PF, Aldrich) were used without further purification. Ascorbate was prepared by addition of sodium hydroxide to an ascorbic acid 10 mM solution. D_2O 99% (Aldrich) was used as received. All other materials were of the best analytical grade available.

Methods

Cells of non-sulfur purple bacteria *Rhodobacter sphaeroides* wild type, were grown under anaerobic conditions in a luminostat at a temperature of 30°C for 5–6 days. Cells were disrupted by sonication. Photosynthetic membranes (chromatophores) were isolated by centrifugation and incubated for 30 min at 4°C in 0.01 M sodium phosphate buffer (pH 7) containing 0.5% zwitterionic detergent Lauryl dimethylamine oxide (LDAO). After the incubation, chromatophores were centrifuged at 144,000*g* for 90 min at 4°C. The supernatant fraction containing RCs was separated chromatographically on a column with hydroxyapatite as described before in more detail (Zakharova and Churbanova 2000). RCs obtained were transferred via dialysis to 10 mM Na-P buffer with 0.1% of anion-active detergent Na-cholate, pH 8.0. RCs in buffer with Na-cholate were more stable during long keeping. During process of column chromatography pigment-protein light harvesting complex 2 (B800-850) was also collected separately and was dialyzed against 10 mM Tris-HCl buffer, pH 8.0. The RC concentration was measured at 800 nm band given the extinction coefficient 0.288 M^{-1} cm^{-1} (Zakharova and Churbanova 2000).

In the NIR emission experiments photoexcitation of the RCs were performed with laser pulses at 532 nm (10 mJ/pulse, 1–10 Hz). All the NIR emission data reported in the manuscript were acquired at 5 Hz. However, we did not observe significant changes when we tested pulse frequencies of 1 or 10 Hz at the same energy/pulse. The quantity of pulses required for the registration of a kinetic decay was around 1,000. There were two components in the kinetics of dark recovery of photooxidized bacteriochlorophyll in these preparations: fast component with $t_{1/2} \sim 100$ ms and amplitude of 60% and slow component with $t_{1/2} = \sim 1$ s and amplitude of 40%. At 5 Hz it is possible to estimate that the amount of RCs with constantly reduced Q acceptor and consequently with constantly oxidized P, during the timecourse of hundreds pulses is near 15%. Experiments with urea, ascorbate, o-phenanthroline, PF were performed by addition of specific amounts of stock solutions to the RC samples. Because that the protein was extracted and kept in water solutions, the experiments made in D_2O had always a mixture of H_2O and D_2O. The ratio of D_2O to H_2O is reported in each experiment. In the experiments in which changes in the temperature and urea concentrations were performed, different samples (at each temperature and each urea concentration) were prepared and both NIR emission and Tryptophan fluorescence were measured. In the experiments of temperature variations, samples were incubated for 3 min at the specific temperatures, and then brought back to $T = 30$°C for the readings. In the experiment of RC incubations in D_2O, the RC

samples were prepared and sealed. The NIR emission was always compared with a sealed sample of MB in order to avoid day-to-day variations in the instrument response. In the Tryptophan emission experiments excitation was performed at 295 nm and fluorescence emission was measured from 305 to 400 nm.

For the calculation of the singlet oxygen quantum yield (ϕ_Δ), absorbance of sample and standard were matched at the excitation wavelength. Because $^1O_2$ lifetime affects the ϕ_Δ determinations it is necessary to keep also the same solvent or to use solvents in which $^1O_2$ has the same lifetime (Wilkinson et al. 1993). Under these conditions, in which sample and standard have the same absorption factor and the same $^1O_2$ lifetime, the maximum intensities of NIR emission are directly proportional to the quantum yields and simple proportionality equations can be written relating emission intensities and ϕ_Δ of sample and standard (Wilkinson et al. 1993). MB is a good standard for ϕ_Δ in polar solvents (Tanielian and Wolff 1995); however, it presents aggregation in aqueous solution (Junqueira et al. 2002). Therefore, we initially calibrated the value of ϕ_Δ of MB in aqueous solution under the experimental conditions used in this work, by comparing the emission of two solutions of MB with the same optical density at 532 nm one prepared in methanol (there is no aggregation and $\phi_\Delta = 0.52$) and another in D_2O:H_2O mixture that matched the lifetime of $^1O_2$ in methanol, i.e., ~ 10 μs (Wilkinson et al. 1995). After the ϕ_Δ value of MB in aqueous solution was obtained, it was used to determine the ϕ_Δ of *Rhodobacter sphaeroides* RCs by comparing emission transients obtained under exactly the same experimental conditions in D_2O:H_2O (9:1). Corrections using the square of the refractive indexes were applied (Demas and Crosby 1971) but changed the results in less than 0.5%. The quantum yield and standard deviation determinations were performed with five independent measurements. In some other experiments, in which the generation of $^1O_2$ was compared, i.e., as a function of ascorbate and potassium ferricyanide concentrations, the absorption at 532 nm changed slightly. In order to compare generation of $^1O_2$ on those conditions, the emission values at 1,270 nm were corrected by the respective absorption factors at 532 nm (Demas and Crosby 1971).

Instrumentations

Absorbance spectra were recorded on a Shimadzu UV-VIS 2400-PC spectrophotometer. Fluorescence spectra were recorded in a SPEX FLUOROG in right-angle mode interfaced to a PC, controlled by DM3000-F software. Some spectral data were further manipulated with a 386 GRAMS software (Galatica, Inc., Salem, NH). For the NIR emission measurements a recently developed instrument was used, whose details have been published before (Gabrielli et al. 2004; Severino et al. 2003). Basically a Nd:YAG laser from *Continuum* (Surelite III, 10 ns light pulses with frequency varying from 1 to 10 Hz) excites the sample into a time-resolved fluorometer (*Edinburgh Analytical Instruments)*, which is connected to a NIR-PMT (R5509 from *Hamamatsu Co*). The emission wavelength was selected using a silicon cutoff filter and a monochromator. The equipment is calibrated weekly and checked for expected emission spectra and lifetime of $^1O_2$ in different conditions.

Results and discussion

Absorption spectra of photosynthetic RC preparations in the visible and NIR regions are shown in Fig. 1. The Q_Y bands at 860 nm, 800 nm and 760 nm are due to P, BChls and BPhs, respectively. Urea caused small changes in the RC absorption spectra while 5 mM potassium ferricyanide (PF) caused P oxidation and consequently a decrease in its characteristic absorbance in 860 nm (Fig. 1A). Addition of sodium ascorbate up to 1 mM did not cause any change in the RC spectra (data not shown). The absorbance spectrum of the antenna-complex solution, which was used as one of the controls in the $^1O_2$ emission experiments, is also presented. The effect of temperature raise in the RC absorbance can be observed in Fig. 1B. Note that there are small changes up to 50°C. At 55°C there is an increase in BPh absorbance with the consecutive decrease in absorbance due to BChls. Although, there are visual changes in the absorbance of P with the temperature increase, when the baseline variations are discounted, the absorbance band did not show representative changes.

Upon photoexciting RCs a transient emission with maximum centered at 1,270 nm was observed (Fig. 2A), which was suppressed by 1 mM azide (Fig. 2A, insert) and showed no change in the presence of o-phenanthroline (Fig. 2A, red curve in the insert). The NIR emission centered at 1,270 nm is the fingerprint of the $O_2(a^1\Delta g) \rightarrow O_2(X^3\Sigma g^-)$ transition and the suppression by sodium azide is in agreement with this designation (Knox et al. 2005; Gabrielli et al. 2004; Severino et al. 2003; Niedre et al. 2006; Tanielian and Wolff 1995) showing that RCs of *Rb. sphaeroides* wild type produces $^1O_2$. Ortho-phenanthroline blocks Q_A–Q_B electron transfer and avoids the generation of reactive oxygen species by the quinone reactions in the acceptor side of the RC (Winfried and Breton 1991). The fact that no change in the $^1O_2$ transient was observed by adding o-phenanthroline indicates that $^1O_2$ generation is not related to the Q_A–Q_B electron transfer reactions. No emission was observed upon photoexciting antenna complexes of *Rb. sphaeroides*. Rinalducci reported $^1O_2$ generation in trimer antenna complexes of plants but

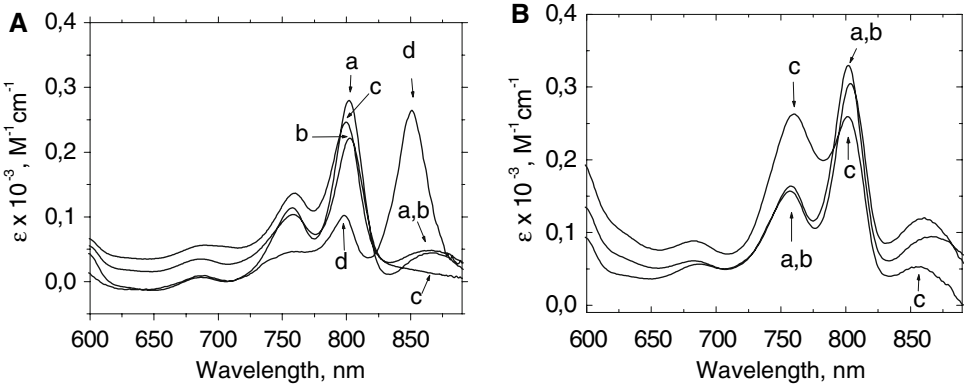


Fig. 1 **A** Absorption spectra of RCs at room temperature in water (*a*), in the presence of 6 M Urea (*b*), 5 mM $K_4(FeCN_6)$ (*c*), and absorption spectra of *Rb. sphaeroides* antenna complexes in water at room temperature (*d*). **B** *Rb. sphaeroides* RCs in water at 30°C (*a*) and treated during 3 min at 50°C (*b*) and 55°C (*c*) and equilibrated to the temperature of 30°C before the measurements

not from monomers, in agreement with our observations (Rinalducci et al. 2004). Emission transients from RCs were obtained both in D_2O:H_2O (9:1) and in H_2O (Fig. 2B, insert) and fitted to single exponential functions yielding lifetimes of 38 μs and 3.5 μs in these two solvents, respectively. The different values of $^1O_2$ lifetimes are expected in these isotropic solvents (Wilkinson et al. 1995), indicating that $^1O_2$ diffuses out of the protein environment and experiences the effect of the surrounding media. Arellano et al. (Arellano et al. 2007) observed practically the same values of $^1O_2$ lifetimes in suspensions of carotenoidless RC of *Rb. sphaeroides*, suggesting that carotenoids do not play a role in the suppression of $^1O_2$ that diffuses out of the protein environment.

Emission intensities captured just after the laser pulse were calculated from solutions of MB in methanol and in a D_2O:H_2O mixture that was adjusted to allow $^1O_2$ to have the same lifetime as the lifetime of $^1O_2$ in methanol, i.e., 10 μs (Fig. 2B). The Φ_Δ of $^1O_2$ generation of MB in methanol is 0.52 (Wilkinson et al. 1993). In aqueous solution, because of the formation of MB dimer species, Φ_Δ is smaller (Junqueira et al. 2002). Comparing both transients in Fig. 2B (a and b) the Φ_Δ value of MB was calculated to be 0.37 in aqueous solution under this experimental condition. Emission transients of RCs and MB, both in D_2O:H_2O (9:1) (Fig. 2B), were acquired under the same experimental conditions and the maximal emission intensities were obtained. By using these emission intensities and the value of 0.37 for the Φ_Δ of MB, it was possible to calculate that the quantum yield of $^1O_2$ generation from *Rb. sphaeroides* RCs is 0.03 ± 0.005. This quantum yield is smaller than those calculated for carotenoidless mutant R-26 (0.09 ± 0.04) (Liu et al. 2005; Arellano et al. 2007) and for PSII of plants ($\Phi_\Delta \sim 0.2$) (Telfer et al. 1994). This fact is in

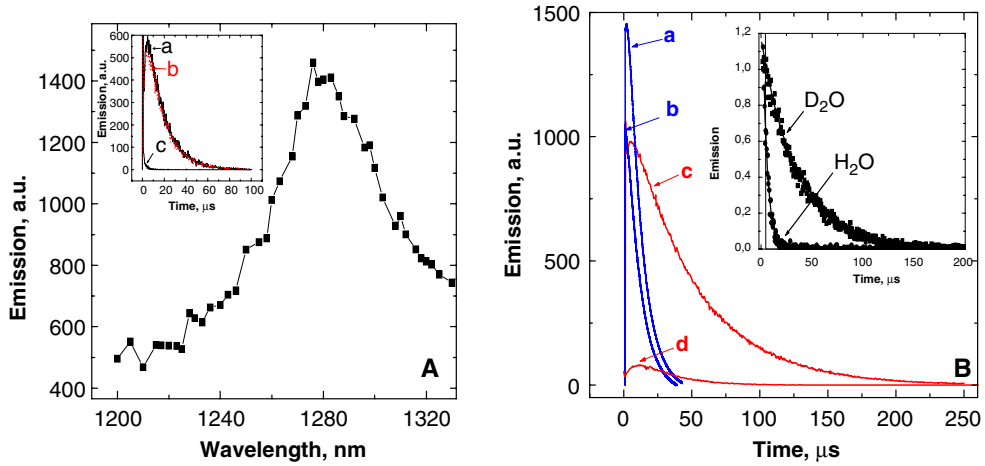
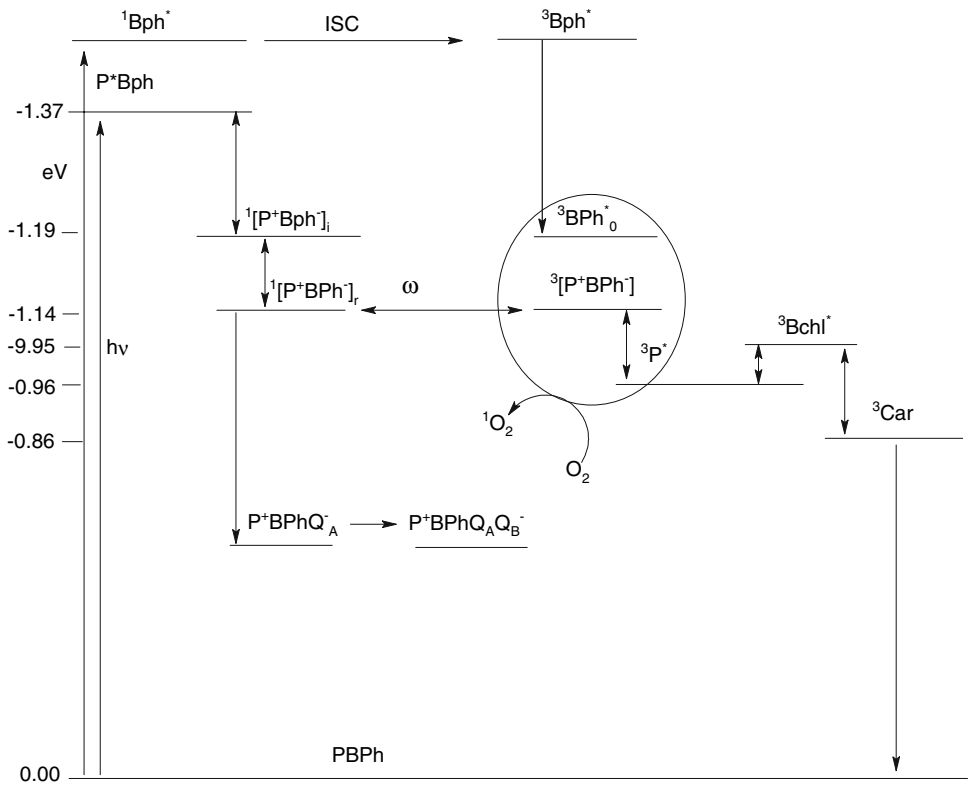


Fig. 2 **A** Emission spectra of *Rb. sphaeroides* RCs in D_2O:H_2O (9:1). Insert: Emission transient of *Rb. sphaeroides* RCs in D_2O:H_2O (9:1) (*a*), in the presence of 1 mM orto-phenanthroline (*b*), and in the presence of sodium azide 1 mM (*c*). **B** Emission transient of MB in methanol (*a*) and in D_2O:H_2O mixture to match lifetime of $^1O_2$ in methanol (*b*) with absorbance equal to 0.26 at 532 nm. Emission transient of MB (*c*) and of *Rb. sphaeroides* RCs (*d*) in D_2O:H_2O (9:1) with absorbance equal to 0.25 at 532 nm. One set of transients is shown but calculations were based on five independent measurements. Insert: Emission transient of *Rb. sphaeroides* RC solutions in water and D_2O:H_2O (9:1). Decays were fitted to monoexponential functions. [RCs] = 2 μM. λ_{exc} = 532 nm. All transients were obtained at 1,270 nm



Scheme 1 Simplified photocycle of *Rb. sphaeroides* RCs where P, BChl , BPh, Q and Car are photoactive dimer, bacteriochlorophyll, bacteriopheophytin, quinone and carotenoid, respectively, $^1[P^+BPh^-]_{i,r}$ are the initial and relaxed states of primary ion-radical pair, respectively, ω is the process of singlet-triplet mixing, ISC is intersystem crossing, 1 and 3 superscripts designate singlet and triplet species, respectively

agreement with the smaller tendency of photoinhibition in wild type *Rb. sphaeroides* compared with plants and also with carotenoidless strains of purple bacteria (Knox and Dodge 1985; Hideg et al. 2000, 2006, 2007; Macpherson et al. 1993, Telfer et al. 1994; Tandori et al. 2001; Liu et al. 2005; Arellano et al. 2007). Three percentage is also much smaller than the Φ_Δ of free Bchl monomers, which is 0.65 for $Bchl_e$ (Arellano et al. 2002). The small Φ_Δ in RCs is compatible with the fact that dimers of Bchl, as well as of other photosensitizers, usually do not generate $^1O_2$ (Gabrielli et al. 2004; Severino et al. 2003; Junqueira et al. 2002; Arellano et al. 2002).

Considering the similarity between *Rb. sphaeroides* RCs and PSII of plants, we believe that the main source of $^1O_2$ generation in these RCs is also the process of P^+BPh^- recombination. Due to the singlet-triplet mixing (ω), there is some probability for the transition of radical pair P^+BPh^- to triplet state and thus to $^3P^*$ formation (Scheme 1) (Shopes and Wraight 1987; Volk et al. 1993; Paschenko et al. 2003). An experimental evidence of the involvement of triplet species in the generation of $^1O_2$, is the effect of sodium ascorbate (asc) (Fig. 3A). It can be observed that with the increase in the asc concentration, there is a decrease in the generation of $^1O_2$ with no change in the $^1O_2$ lifetime. Ascorbate is a reducing agent and an efficient triplet suppressor. The redox suppression of triplets decreases the quantum efficiency of energy transfer to oxygen, proving the involvement of triplets in the generation of $^1O_2$.

Evidences for the role of $^3P^*$ in the generation of $^1O_2$ were obtained by asking initially how the dynamics of the electron transfer reactions in the RCs interfere in the generation of $^1O_2$ and subsequently, how the NIR emission from the RCs is changed in the absence of active P. RCs incubation in D_2O during several days causes Hydrogen to Deuterium isotope substitution, lowering the efficiency of electron transfer reactions, increasing the lifetimes of the intermediate species and consequently favoring the formation of $^3P^*$ (Cioni and Strambini 2002; Paschenko et al. 1998). There is a fivefold increase in the $^1O_2$ emission during the 5-day experiment without lifetime change, suggesting the main role of $^3P^*$ species in the generation of $^1O_2$ (Fig. 3B). The role of $^3P^*$ was confirmed by P oxidation promoted by addition of potassium ferricyanide. With the oxidation of P there is a threefold decrease in $^1O_2$ emission proving the involvement of P triplets in this process (Fig. 3C). However, even after the addition of 5 mM PF, which far exceeds the concentration needed to totally oxidize P, there is still $\sim$30% of the $^1O_2$ emission remaining (Fig. 3C), suggesting that there are other sources of $^1O_2$, which are not related to P.

$^3P^*$ molecules are especially reactive and the main role of Car is to quench them before they photosensitize the $^1O_2$ formation (Krieger-Liszkay 2004). In *Rb. sphaeroides* RCs, Car is located within van der Waals distance of $BChl_B$ ($\sim$3.7 Å) and at 10 Å of P (Arnoux et al. 1995). It has been proposed that triplet transfer involves $BChl_B$ as

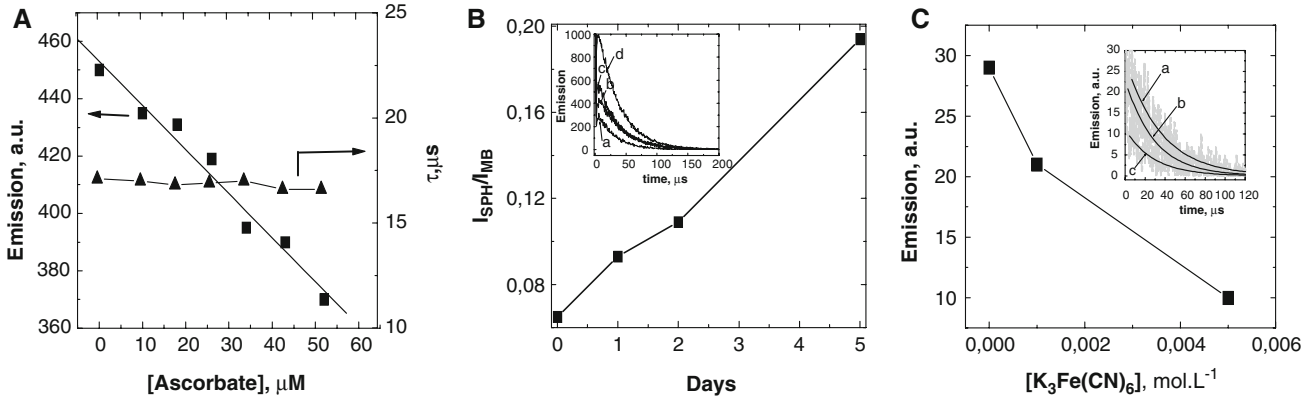


Fig. 3 **A** Emission intensity (*left axis*) from *Rb. sphaeroides* RCs and $^1O_2$ lifetime (*right axis*) as a function of sodium ascorbate concentration in D_2O:H_2O (8:2). **B** Relative emission intensity as a function of incubation time in D_2O. Insert: Emission transients at 0 (*a*), 1 (*b*), 2 (*c*), and 5 (*d*) days. [RCs] = 2 μM; λ_{exc} = 532 nm. **C** Emission intensity from *Rb. sphaeroides* RCs as a function of potassium ferrocianide concentration. Insert: Emission transients from *Rb. sphaeroides* RCs at different $K_3Fe(CN)_6$ concentrations: 0 (*a*), 0.001 (*b*) and 0.005 (*c*) mol L^{-1}. In **B** and **C** D_2O:H_2O was 9:1. Transient emissions were always obtained at 1,270 nm

a bridging molecule (Frank et al. 1993). However, because of the close proximity to Car it is unlikely that BChl triplets will live long enough to react with molecular oxygen. Under 532-nm illumination, BPh is directly excited. In functionally active RCs, excitation energy migrates quickly to P and thus is deactivated effectively in the process of charge separation (Paschenko et al. 1998). Under high energy and frequency light pulses, BPh molecules can form triplet species. Contrary to BChl, BPh molecules are situated beyond the Van der Waals contact sphere of Car. Therefore, BPh triplets are another possible source of $^1O_2$.

By heating RCs, the photoactive pigments are exposed promoting their pheophytinization, which can be followed by the increase in the characteristic BPh absorption band at 760 nm (Fig. 1B). Note an increase in BPh absorption with a consecutive decrease in BChl absorption. The heating is accompanied by a four-time increase in the $^1O_2$ emission (Fig. 4A, filled square), suggesting that BPh triplets may indeed be the other source of $^1O_2$ generation. It is important to notice that $^3BPh^*$ is an efficient generator of $^1O_2$ in solution ($\Phi_\Delta = 0.75$) (Krasnovsky et al. 1993).

It is also important to consider that the temperature increase affects RCs structure, as confirmed by a decrease in the Tryptophan fluorescence (Fig. 4A, filled circle). This change is not a complete denaturation, since the wavelength of Tryptophan emission maximum does not change during the experiment. Nevertheless, it is imperative to test which effect, i.e., change in the protein structure or pheophytinization, is the responsible for the increase in $^1O_2$ generation. Urea is known to affect protein structure without causing pheophytinization. Tryptophan fluorescence and NIR emission of RCs were also obtained as a function of urea concentration (Fig. 4B). Urea causes a similar change in the Tryptophan environment as observed by heating, without causing pheophytinization (Figs. 1B, 4B, filled circle). Under this condition, the NIR emission decreases (Fig. 4B, filled square) instead of increasing, confirming that the increase of $^1O_2$ emission observed by heating RCs is due to the increase in $^3BPh^*$. Therefore, we have shown that part of the $^1O_2$ generated in the RCs indeed comes from type II photosensitization of $^3BPh^*$ without involvement of P. In plants, it is believed that the other possible source of $^1O_2$ is through direct excitation of

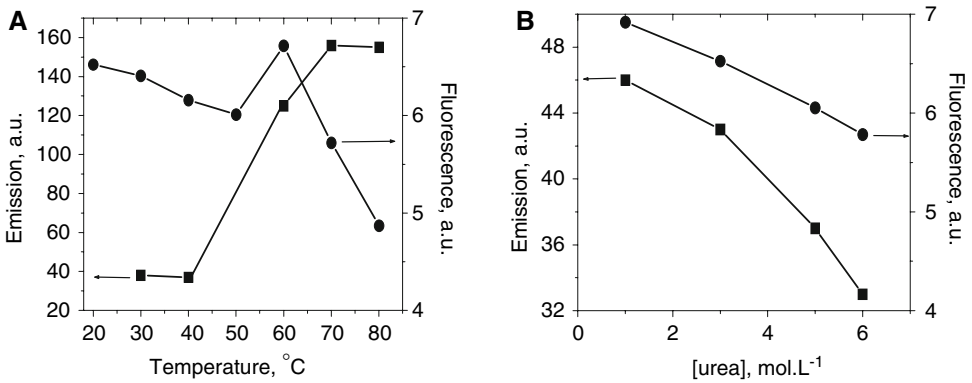


Fig. 4 *Left axis*: emission at 1,270 nm (λ_{exc} = 532 nm), *right axis*: Tryptophan fluorescence (λ_{exc} = 295 nm, λ_{em} = 305–400 nm) of *Rb. sphaeroides* RCs as a function of temperature of incubation (**A**) and urea concentration (**B**). [RCs] = 2 μM, D_2O:H_2O was 9:1. All transients were obtained at T = 30°C

Chlorophyll leading to triplets and type II photosensitization (Krieger-Liszkay 2004), which is in accordance with our observations in *Rb. sphaeroides* RCs (Scheme 1).

Conclusions

We have shown that the RCs of *Rb. sphaeroides* wild type under photon overload conditions produce $^1O_2$ with quantum yield of 0.03 ± 0.005. This is the first report in the literature showing that RCs of *Rb. sphaeroides* wild type generates $^1O_2$. Under this condition the formation of $^3(P^+BPh^-)$ and $^3P^*$ surpass the protection by the energy transfer to ^{3}BCh and ^{3}Car and $^1O_2$ is formed (Scheme 1). The other source of $^1O_2$ was shown to be due to the formation of $^3BPh^*$ produced by photoexcitation of BPh. Quantifying and understanding the molecular mechanisms of $^1O_2$ production are important steps in trying to control photoinhibition.

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