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Transient optical spectroscopy of single crystals of the reaction center from *Rhodobacter sphaeroides* wild-type 2.4.1

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The photoactivity of the crystallized reaction centers from *Rhodobacter sphaeroides* wild-type strain 2.4.1 has been examined by light-induced absorption spectral changes associated with charge separation and triplet state formation in the reaction center. Upon excitation of a crystal at ambient redox potential, the primary donor 865 nm band bleaches reversibly. The kinetics of its recovery were found to be biphasic with rate constants $11.5 \pm 1.3\ s^{-1}$ and $0.9 \pm 0.4\ s^{-1}$ which correspond to lifetimes of 87.0 ± 9.0 ms and 1.0 ± 0.7 s, respectively. The ratio of the fast-to-slow component preexponential terms was 3.5 ± 1.1 suggesting that the majority ($78.9 \pm 13.0\%$) of the reaction centers in the crystals lack the secondary quinone, Q_B. The addition of sodium ascorbate to the crystals attenuates the 865 nm absorption change, and gives rise to strong carotenoid triplet–triplet absorption changes at 547 nm. These data indicate that the reaction center-bound carotenoid in the crystals is capable of accepting triplet energy from the primary donor triplet.

Introduction

The correlation of the spectroscopic observables arising from the protein-bound pigments in the photosynthetic bacterial reaction center with the molecular structures provided by X-ray analyses [1–7] is of major importance in understanding the photosynthetic process. Once done, it should be possible to pinpoint specific structural features which have a bearing on the rates and efficiencies of the primary charge separation and triplet state formation in the reaction center. In order to make these correlations, it is distinctly advantageous to carry out the spectroscopic experiments directly on the crystalline samples used in the X-ray diffraction analyses. In this manner the most direct correlations between the structure of the reaction center and its photochemical properties will emerge. Also, because the crystallization process could alter the structure of the reaction center protein, it is important to develop methods which will allow a continuous assessment of the integrity of the photochemical processes carried out by the complex in crystalline form.

The organization of the reaction center pigments in the carotenoid-containing photosynthetic bacterial reaction centers has been determined by Deisenhofer et al. for *Rhodopseudomonas viridis* [1–3] and by Allen et al. for *Rhodobacter sphaeroides* strain 2.4.1 [5,6]. There is an approximate two-fold rotation symmetry which relates the bound bacteriochlorophylls, bacteriopheophytins and quinones. Also, both *Rps. viridis* and *Rb. sphaeroides,* have a bound carotenoid molecule which occurs in a 1 : 1 stoichiometric ratio with the primary donor [8,9]. This molecule is the only chromophore in the reaction center that does not adhere to the approximate two-fold rotation symmetry. The most recent coordinate refinement of the X-ray data carried out by Allen et al., [5,6] on reaction centers from the 2.4.1 strain has located the reaction center carotenoid between the B and C helices of the M protein subunit and near the associated monomeric accessory bacteriochlorophyll which lies between the carotenoid and the primary donor. This is similar to the *Rps. viridis* carotenoid position which was found approx. 1.08 nm from the primary donor [3]. The X-ray determined structures are not yet sufficiently resolved to assign unambiguously the stereochemistry of the carotenoids.

Recently, electron spin resonance (ESR) experiments have been performed on *Rb. sphaeroides* 2.4.1 reaction center crystals [10]. The ESR experiments were done in the temperature range 10–70 K. These studies demonstrated that the crystals were capable of carrying out the

Abbreviations: LDAO, lauryldimethylamine *N*-oxide; *o*-phen, *o*-phenanthroline; UQ, ubiquinone-50; ESR, electron spin resonance.

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reaction center photochemistry. In addition, pronounced anisotropy in the primary donor triplet state ESR spectra was observed. The studies revealed the explicit orientations of the magnetic axes of the four primary donor molecules with respect to the unit cell axes. However, conspicuously absent from the ESR spectra taken at temperatures above 35 K were carotenoid triplet state signals. In reaction centers of *Rb. sphaeroides* 2.4.1 the triplet state of the carotenoid, spheroidene, is known to quench the primary donor triplet state with almost unity quantum yield at temperatures above 35 K [11,12]. This normally gives rise to strong carotenoid ESR signals from reaction centers in solution [9]. Given that the carotenoid is indeed present in the crystals (the crystals have the distinctive reddish-brown color indicative of the presence of carotenoids, and the absorption spectrum of the crystals has been previously reported [13] showing peaks attributable to the bound carotenoid) there are two plausible explanations for the lack of ESR-observable carotenoid triplet states: (1) the crystallization process has affected the ability of the carotenoids to form triplets. This would most likely come about from a change in the structure or geometry of the primary donor-carotenoid, donor-acceptor pair which could change the efficiency of triplet energy transfer; and (2) disorder in the carotenoid structure in the crystal is sufficiently high that the carotenoid triplet state signals cannot be observed. The primary donor triplet state signals on the other hand are very strong owing to that molecule's high degree of structural order.

To determine whether or not the carotenoid triplet is indeed capable of quenching the primary donor triplet, transient optical spectroscopy was employed directly on the single crystals. This technique (using unpolarized light) is less constrained by orientation factors than ESR. Also, these studies provide an assay of the photochemical activity of the crystals and a measure of the quinone content of the crystals via the dynamics of the recovery of the absorbance changes associated with the primary charge separation.

Materials and Methods

The reaction centers were prepared according to the following procedure: *Rb. sphaeroides* 2.4.1 cells were grown anaerobically in modified Hutners media [14]. Chromatophores were obtained by French pressure disruption at $1.4 \cdot 10^8$ Pa of whole cells followed by ultracentrifugation at $150\,000 \times g$ for 90 min. The chromatophore membranes were stirred in 15 mM Tris buffer (pH 8.0)/0.3% LDAO at 5°C overnight. NaCl (final concentration of 150 mM) was then added to the mixture which was incubated at 28°C for 30 min. The mixture was then centrifuged at $12\,000 \times g$ for 15 min to remove the membrane debris. The supernatant from the low spin was centrifuged at $250\,000 \times g$ for 60 min. The supernatant enriched in reaction centers was diluted 1:4 with 15 mM Tris buffer (pH 8.0)/0.1% LDAO and loaded onto a 3×20 cm DEAE Sephacel (anion exchange, Sigma No. I-6505) which was previously equilibrated with 500 ml of 15 mM Tris buffer (pH 8.0). The protein fractions were eluted from the column by a step gradient elution using 15 mM Tris buffer (pH 8.0)/0.2% LDAO/0.04–0.20 M NaCl solutions in 0.02 M NaCl concentration steps. The reaction center protein fractions were obtained at 0.18 M NaCl concentration. The fractions ($A_{280}/A_{800} = 2.1$) were then diluted 1:4 with 15 mM Tris buffer (pH 8.0)/0.1% LDAO and loaded onto a second 3×20 cm DEAE Sephacel column. The reaction centers were washed with 15 mM Tris (pH 8.0)/0.2% LDAO/0.06 M NaCl until the remaining free protein was removed. The purified reaction centers ($A_{280}/A_{800} = 1.2$) were obtained by elution with 15 mM Tris buffer (pH 8.0)/0.2% LDAO/0.35 M NaCl. Finally, the purified reaction centers were loaded onto a third 1×5 cm Sephacel column and washed with 15 mM Tris buffer (pH 8.0) containing 0.8% β-octylglucoside ($30 \times$ bed volume) to exchange the detergent. The purified reaction centers in β-octylglucoside were eluted with 15 mM Tris buffer (pH 8.0)/0.8% β-octylglucoside/0.35 M NaCl.

Crystallization of the *Rb. sphaeroides* 2.4.1 reaction centers was accomplished by vapor diffusion at 22°C in the dark. 50 µl droplets containing 44 µM reaction centers, 0.8% β-octylglucoside, 10% (w/v) PEG 4000, 2% heptanetriol, 0.12 M NaCl, 15 mM Tris buffer at pH 8.0, 1 mM EDTA and 0.1% NaN_3 were equilibrated against 22% (w/v) PEG 4000, 0.24 M NaCl, 15 mM Tris buffer at pH 8.0, 1 mM EDTA, 0.1% NaN_3. After about 4 weeks at room temperature, crystals with typical dimensions of 1.5 mm × 0.05 mm × 0.05 mm were obtained.

Ubiquinone-50 (UQ-) reconstituted reaction centers were prepared by a modification of method previously described [15]. A 40-fold excess of UQ in 50 µl of ethanol was added to 1 ml of 3.4 µM reaction center solution. The reaction center-UQ mixture was gently stirred at room temperature, in the dark, for 16 h, and then clarified by passage through a 0.45 µm Cameo membrane filter. 100 µl of *o*-phenanthroline (*o*-phen) was added to 1 ml of the UQ-reconstituted reaction center samples from 100 mM stock solution in ethanol to give a final *o*-phen concentration of 10 mM. The samples were then gently stirred for 2 h prior to the optical experiments.

The optical absorbance changes were measured at room temperature in the following way: Light from an 80 W Xe arc lamp was filtered through 3.5 cm of water in a Pyrex bottle and rendered monochromatic by passing through a Kratos model GM252 monochromator. The light beam was focussed onto the crystal using a

Leitz-Wetzlar model 559-123 UT 40/0.35 ultra-long working distance microscope objective mounted on a micrometer translation stage. The crystal in mother liquor was sealed in a microcell with a 10 μm spacer between two cover slips. The light transmitted through the crystal was focussed by another identical objective through an appropriate interference filter (e.g., 865 nm or 547 nm) onto an EG & G model UV-0.40 BG high sensitive photodiode detector. The output was amplified using either an Evans Associates model 4163 wide band (d.c. to 10 MHz) amplifier for the microsecond time-resolved experiments or a current amplifier of local design for the millisecond time-resolved experiments and fed to a Nicolet LAS 12/70 transient digitizer. The intensity of the measuring beam was kept low enough to prevent photooxidation of the reaction centers during the measurements. The flash-induced photochemistry was initiated by a Quanta-Ray DCR3/PDL-2 Nd:YAG-pumped dye laser (pulse duration: 7 ns FWHM; $\lambda = 587$ nm) using dye Rhodamine 590 dissolved in methanol. The repetition rate was 0.2 Hz for the 1 s time profiles and 20 Hz for the 100 μs time profiles. The actinic beam was focussed onto the crystal, made colinear with the measuring beam, and kept below saturation.

Results and Discussion

Photoactivity of crystalline reaction centers

Fig. 1a and b shows the kinetics of flash-induced transmittance changes measured at 865 nm and 787 nm,

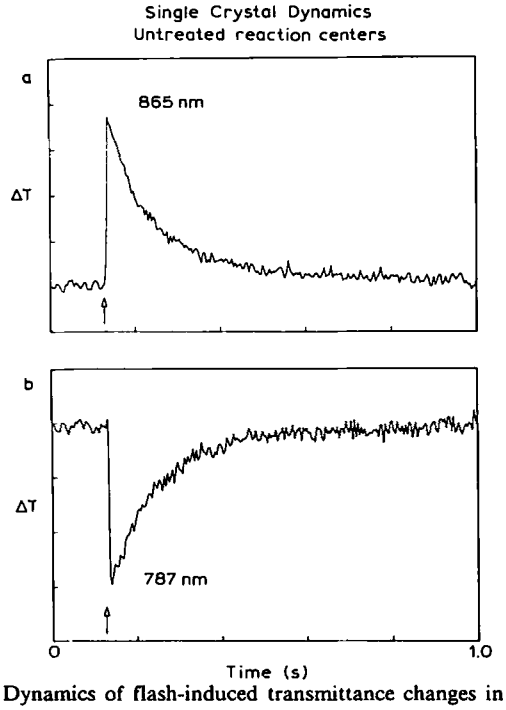

Fig. 1. Dynamics of flash-induced transmittance changes in a single crystal of *Rb. sphaeroides* 2.4.1 reaction centers: (a) 865 nm detection; (b) 788 nm detection. The arrow indicates when the laser flash was applied. Both traces are the average of 200 sweeps.

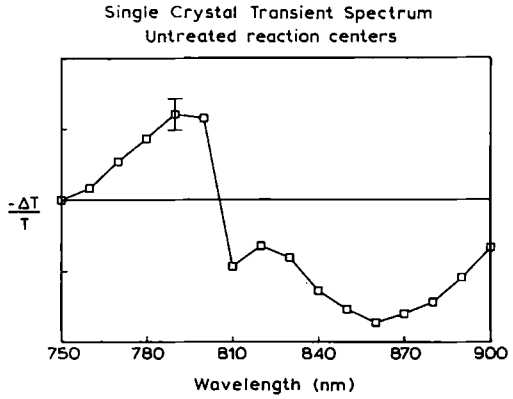

Fig. 2. Spectrum of the flash-induced transmittance changes in a single crystal of *Rb. sphaeroides* 2.4.1 reaction centers. The transmittance changes were normalized to the total transmittance at the specified wavelength and measured at a time concurrent with the laser flash.

respectively, in a single crystal of *Rb. sphaeroides* 2.4.1 reaction centers. The transients were analyzed according to a double exponential rate expression and the kinetic parameters obtained (see Table I). The two components had rate constants of 11.5 ± 1.3 s^{-1} and 0.9 ± 0.4 s^{-1} which correspond to lifetimes ($\tau = 1/k$) of 87.0 ± 9.0 ms and 1.0 ± 0.7 s, respectively. The ratio of the (fast-to-slow component) preexponential terms is 3.5 ± 1.1 indicating that the fast component dominates the decay. Fig. 2 shows the spectrum of the light-induced transmittance changes measured at a time concurrent with the later flash.

The LDAO used in the isolation and purification of reaction centers has been shown to dislodge quinones from the reaction centers [16–19]. To determine the quinone contents of these crystals, three solutions were prepared and analyzed as controls. (1) Reaction center solution purified using the same procedure as those from which the crystals were obtained (see Materials and Methods). (2) The purified reaction center solution to which an excess of UQ was added. (3) The UQ-reconstituted reaction center solution to which *o*-phen was added. *o*-phen is known to block the electron transfer from Q_A to Q_B [17,18]. Fig. 3a shows the kinetics of the transmittance change at 865 nm in the purified reaction center solution. Once again the data were fit to a double exponential rate expression. The rate constants for the two components are 11.8 ± 0.1 s^{-1} and 1.1 ± 0.5 s^{-1} corresponding to lifetimes of 85.0 ± 0.4 ms and 1.0 ± 0.4 s, respectively. The ratio of the (fast-to-slow component) preexponential terms is 10.8 ± 2.8, indicating, as above, that the fast component dominates. Fig. 3b shows the kinetics of the transmittance changes at 865 nm in the UQ-reconstituted reaction center solution. The double exponential analysis of this trace gave rise to a rate constant of 12.4 ± 0.2 s^{-1} for the fast component and a rate constant of 1.0 ± 0.1

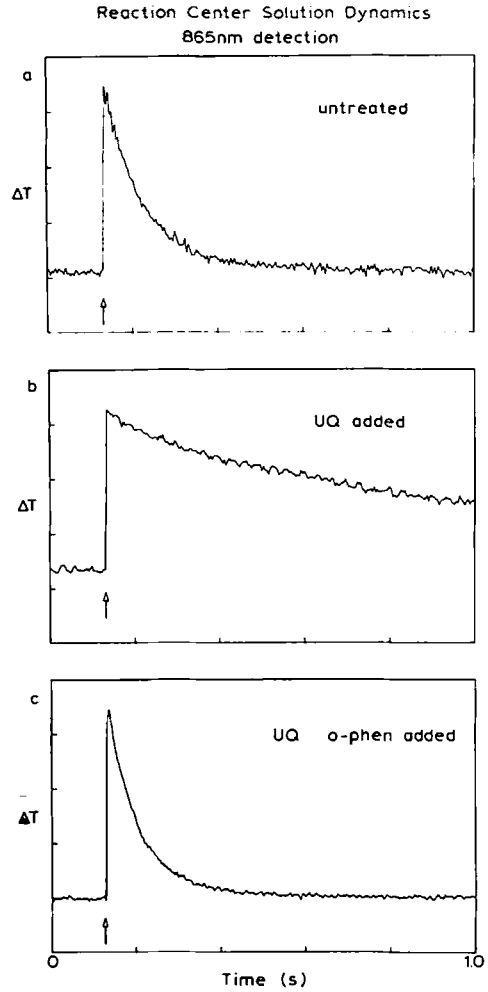

Fig. 3. Dynamics of flash-induced transmittance changes measured at 865 nm in *Rb. sphaeroides* 2.4.1 reaction center solutions: (a) untreated reaction center solution; (b) reaction center solution to which an excess of UQ was added; (c) UQ-reconstituted reaction center solution to which *o*-phen was added. Sample concentration, 3.4 μM reaction centers in 15 mM Tris buffer (pH 8.0), 0.2% LDAO. The arrows indicate when the laser flash was applied. All three traces are the average of 200 sweeps.

s^{-1} for the slow component which correspond to lifetimes of 82.0 ± 8.0 ms and 1.0 ± 0.1 s, respectively. The ratio of the (fast-to-slow component) preexponential terms is 0.18 ± 0.05, indicating here that the slow component dominantes the decay. Fig. 3c shows the kinetics of the transmittance changes at 865 nm in the UQ-reconstituted reaction center solution to which *o*-phen was added. The rate constants for the two components are 14.9 ± 0.5 s^{-1} and 1.0 ± 0.2 s^{-1}. These correspond to lifetimes of 67.5 ± 1.3 ms and 1.0 ± 0.2 s, respectively. The ratio of the (fast-to-slow component) preexponential terms is 14.0 ± 5.0 indicating the predominance of the fast component in the decay.

The transient signal observed at 865 nm in solution and the crystal (Figs. 1a and 3a) is well known [17,18] to be the absorption bleaching of primary donor bacteriochlorophyll dimer ($BChl_2$) resulting from its oxidation after a flash. Fig. 1b represents the enhanced absorption at 787 nm due to the flash-induced bacteriochlorophyll band shift [20]. The dynamics of the reaction center solution to which an excess UQ was added display a dominant slow component, having a lifetime of 1.0 ± 0.1 s, characteristic of the charge recombination from $BChl_2^+Q_B^-$ state to $BChl_2Q_B$ state [17,18]. It is also known that when *o*-phen is added to the reaction centers, the electron transfer from Q_A to Q_B is blocked [17,19] and a fast back reaction from the $BChl_2^+Q_A^-$ state is expected to be observed. The dominant fast component with a lifetime of 67.5 ± 1.3 ms observed here (Fig. 3c) is consistent with this charge recombination from $BChl_2^+Q_A^-$ to $BChl_2Q_A$. Thus, the data presented in Figs. 1 and 3 demonstrate that the photochemical behavior of the crystalline sample is essentially identical to that which occurs in solution. Moreover, the spectrum of the absorbance changes in the crystal shown in Fig. 2 is the same as that observed for the reaction centers in solution (see Fig. 3 and Ref. 20). Upon photooxidation of the primary donor ,the 865 nm band bleaches reversibly. In addition, there is a shift

TABLE I

Dynamics of primary donor bleaching recovery in reaction centers of Rhodobacter sphaeroides 2.4.1

The kinetics of flash-induced absorbance changes were measured at 865 nm with laser repetition rate of 0.2 Hz. The transients were analyzed according to the equation $y = A + Be^{-k_1 t} + Ce^{-k_2 t}$. The preexpotential ratio is the ratio of the fast-to-slow component preexponential terms, B/C.

Rb. sphaeroides 2.4.1 reaction center preparation	Dynamics parameters					RC's lacking Q_B activity (%)
	k_1 (s^{-1})	τ_1 (ms)	k_2 (s^{-1})	τ_2 (s)	Preexponential ratio	
Single crystal						
Untreated	11.5±1.3	87.0±9.0	0.9±0.4	1.0±0.7	3.5 ±1.1	78.0±13.0
Solution						
Untreated	11.8±0.1	85.0±0.4	1.1±0.5	1.0±0.4	10.8 ±2.8	90.0±9.0
UQ added	12.4±0.2	82.0±8.0	1.0±0.1	1.0±0.1	0.18±0.05	15.0±2.8
UQ+ *o*-phen added	14.9±0.5	67.5±1.3	1.0±0.2	1.0±0.2	14.0 ±5.0	93.0±6.0

of the 800 nm band to shorter wavelengths giving rise to an absorption increase at 790 nm.

The ratio of the values of the fast component preexponential term to that of the sum of the fast and slow component values in the crystal indicate that 78.0 ± 13.0% of the reaction centers in the crystal lack Q_B (see Table I). It is known [16,17] that the secondary quinone, Q_B, is loosely bound to the protein and is easily lost during isolation and purification of the reaction centers using LDAO [16–19]. The purified reaction centers in solution are shown here to have lost 90.0 ± 9.0% of their Q_B molecules (see Table I). Because this reaction center solution control sample shows a similar loss of Q_B as do the crystalline samples it is concluded that the Q_B molecules were lost during the isolation and purification of the reaction centers.

Primary donor-to-carotenoid triplet energy transfer

Fig. 4a (lower trace) shows the kinetics of microsecond time-resolved flash-induced transmittance changes measured at 547 nm in a single untreated reaction center crystal. Fig. 4a (upper trace) shows the kinetics of flash-induced transmittance change measured at 547 nm in the crystal to which ascorbate has been added. The data from the ascorbate-treated single reaction center crystal at 547 nm were fit to a single exponential rate expression which resulted in a rate constant of

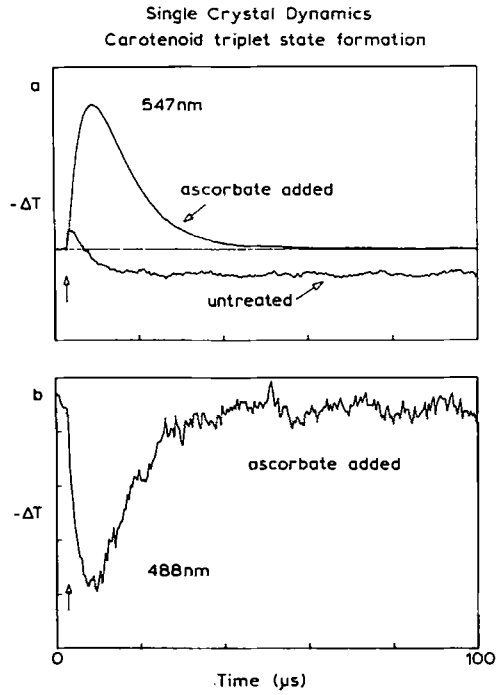

Fig. 4. Dynamics of flash-induced transmittance changes measured in a single crystal of *Rb. sphaeroides* 2.4.1 reaction centers. (a) 547 nm detection; (lower trace) untreated reaction center crystal; (upper trace) reaction center crystal to which sodium ascorbate was added; (b) 488 nm detection; sodium ascorbate added. The vertical arrows indicate when the laser flash was applied. Both traces are the average of 2000 sweeps.

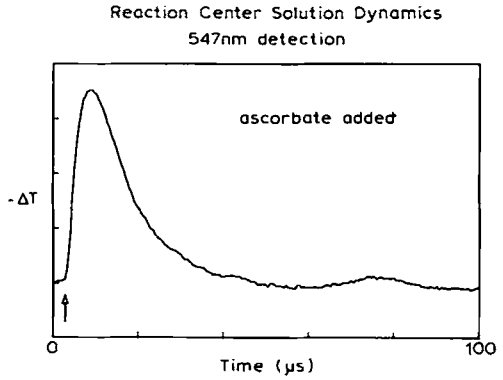

Fig. 5. Dynamics of flash-induced transmittance change measured in a solution of *Rb. sphaeroides* 2.4.1 reaction centers. 547 nm detection; sodium ascorbate added. Sample concentration, 3.4 μM reaction centers in 15 mM Tris buffer (pH 8.0), 0.15% Triton X-100. The arrow indicates when the laser flash was applied. The trace is the average of 2000 sweeps.

$(1.28 \pm 0.03) \cdot 10^5\ s^{-1}$ for the observed transient. This corresponds to a lifetime of the 8.0 ± 0.2 μs. The same dynamics data are observed for the kinetics of flash-induced transmittance changes measured at 488 nm (Fig. 4b) and 520 nm (data not shown).

In order to provide the proper control for determining whether or not carotenoid triplet states are being formed in the crystals, a reaction center solution to which ascorbate was added was analyzed. Fig. 5 shows the kinetics of the flash-induced transmittance changes measured at 547 nm in the ascorbate-treated reaction center solution. The rate constant for this transient is determined to be $(1.33 \pm 0.09) \cdot 10^5\ s^{-1}$ corresponding to a lifetime of 7.5 ± 0.5 μs.

The intense absorption transient observed at 547 nm in the reaction center solution to which ascorbate is added (Fig. 5) is known to arise from the carotenoid triplet state formed as a result of quenching the primary donor triplet [21,22]. In fact, the absorbance change associated with the primary donor bleaching at 865 nm is greatly attenuated in the reaction center solution to which ascorbate has been added (data not shown). The same, intense, 547 nm transient is observed in the ascorbate-treated reaction center crystal (Fig. 4). Thus, the data presented in Figs. 4 and 5 demonstrate that the bound carotenoid, spheroidene, is capable of efficiently quenching the primary donor triplet state in a single crystal of reaction centers from *Rb. sphaeroides* strain 2.4.1. Moreover, the formation of the absorption transient at 547 nm occurs concurrent with an 82.0 ± 2.4% reduction of the primary donor bleaching intensity measured at 865 nm in the same crystal (data not shown). This indicates that the addition of ascorbate, followed by actinic flashes of light, has inhibited the electron transfer beyond the initial electron acceptor similar to that observed in reaction center solution. This is well-known to cause the formation of the primary donor

TABLE II

Dynamics of the carotenoid triplet decay in reaction centers of Rhodobacter sphaeroides 2.4.1

The kinetics of flash-induced changes were detected at 547 nm with a laser repetition rate of 20 Hz. The transients were analyzed according to the equation $y = A + Be^{-kt}$.

Rb. sphaeroides 2.4.1 reaction center preparation	Dynamics parameters	
	k (s^{-1})	τ (μs)
Single crystal	$(1.28 \pm 0.03) \cdot 10^5$	8.0 ± 0.2
Solution	$(1.33 \pm 0.09) \cdot 10^5$	7.5 ± 0.5

triplet state which can then be rapidly quenched by the carotenoid triplet [21,22]. The carotenoid triplet-triplet absorption measured at 547 nm (Fig. 4a) and at 520 nm (data not shown) and the observed bleaching of the ground-state absorption band of the carotenoid at 488 nm (Fig. 4b) in the ascorbate-treated reaction center crystal display the same flash-induced absorbance changes associated with carotenoid triplet state formation in reaction center solutions used here as the control (Fig. 5) and previously reported in the literature [22–24]. The rate constant of the transient measured at 547 nm in the crystal is essentially the same as that in the solution used as control (Table II). The lifetime for the decay of the carotenoid triplet state is measured here to be 7.5 ± 0.5 μs in the solution and 8.0 ± 0.2 μs in the crystal. These values are in agreement with the lifetime reported in the literature for the carotenoid triplet decay [21,22].

The similarity between the dynamics of the primary donor bleaching recovery determined for the reaction center crystal and those in the solution along with the observation of an intense carotenoid triplet–triplet absorption in the crystal indicate clearly that both the electron transfer from $BChl_2$ to Q_A and triplet–triplet energy transfer from $BChl_2$ to the carotenoid are unimpaired in single crystals of *Rb. sphaeroides* 2.4.1 reaction centers and essentially identical to those observed in solution. The fact that no carotenoid triplets were observed in the single crystal ESR experiments [10] previously alluded is therefore not due to the crystallization process rendering the carotenoid incapable of performing its photochemical role of quenching the primary donor state. Rather, it is most likely attributable to partial disordering of the carotenoid molecules. Indeed, the disorder must be only parital, since the reaction center crystals exhibit linear dichroism in the carotenoid spectral region [13]. Also, a high degree of carotenoid ordering has been suggested from previous photoselection [25] and magnetophotoselection [26] studies carried out on *Rb. sphaeroides* 2.4.1 reaction centers in solution. Because a typical sample volume of a single crystal used in the ESR experiment is about 30000 times less than that of a non-crystalline sample [27], a partially disordered triplet would be difficult to detect by standard ESR methods. The lack of a well-defined electron density map for the carotenoid molecule in the reaction centers of *Rps. viridis* and *Rb. sphaeroides* could well be taken as a suggestion that the carotenoid is structurally disordered consistent with the foregoing discussion.

Acknowledgements

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