Volume 239, number 1, 78–82 FEB 06425 October 1988

Spectroscopic characterization of reaction center crystals from the carotenoid-containing wild-type strain *Rhodobacter sphaeroides* Y

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Received 30 August 1988

Thin three-dimensional crystals of the photochemical reaction center from *Rhodobacter sphaeroides* wild-type strain Y were investigated with microspectrophotometric methods. Absorbance and linear dichroism spectra were obtained for two crystal forms with the same type of unit cell, but grown in different morphological forms. High dichroism was found for the Q_x and the Q_y transitions of the bacteriopheophytin as well as for the Q_y transitions of the accessory bacteriochlorophylls and the bacteriochlorophylls forming the primary electron donor. Low absorption and dichroism of the carotenoid transitions in the plane of the crystal suggests a large component out of the crystal plane. Photoactivity of the crystal was demonstrated by light-induced charge separation. The analysis of the rates of charge recombination indicates that almost all reaction centers in the crystal (>90%) contain the secondary electron acceptor Q_B.

Photochemical reaction center; Membrane protein crystal; Microspectrophotometry; Linear dichroism; Carotenoid

1. INTRODUCTION

Reaction centers of photosynthetic organisms are membrane-bound pigment-protein complexes that are able to convert light energy into a charge separated state of an electron donor and an electron acceptor molecule. Due to recent progress in their crystallization, the structures of reaction centers from two purple bacteria strains, *Rhodopseudomonas viridis* [1–3] and *Rhodobacter sphaeroides* R26 [4–7] have been solved at high resolution; they show considerable homology. Reaction centers from another *Rb. sphaeroides* strain (Y) are currently studied by X-ray diffraction [8,9]. They differ from those of the R26 mutant in a few but important properties. Unlike the carotenoidless mutant R26, they contain a firmly bound spheroidene molecule, which is a *cis*-isomer [10,11]. In *Rp. viridis*, as in *Rb. sphaeroides* R26 reaction centers, the stable electron acceptor is a Fe^{2+}-quinone(s) complex. In *Rb. sphaeroides* Y the predominant metal bound to the quinones is Mn^{2+} [12,13], and can be exchanged biosynthetically for other metals such as Fe^{2+} [13], Co^{2+} and Zn^{2+} (Agalidis, I., Rutherford, W.A. and Reiss-Husson, F., unpublished) and Cu^{2+} [14]. Crystals of the *Rb. sphaeroides* Y RC have been shown to contain spheroidene and 1 Mn^{2+} per RC [8].

Here we report on the polarized absorption spectra of *Rb. sphaeroides* Y RC single crystals, in an attempt to get information on the pigment orientation. Photochemical activity of the RC in the crystalline form is demonstrated by light-induced charge separation. The dynamics of charge recombination shows that the RC crystals contain the two quinones Q_A and Q_B in a fully functional state.

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Abbreviations: BChl a, bacteriochlorophyll a; BPheo a, bacteriopheophytin a; Q_A, Q_B, quinone A, quinone B; RC, reaction center; P, primary electron donor

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

2.1. *Crystallization*

Crystallization of the *Rb. sphaeroides* Y RC was performed as described in [8,9] at 18°C. Orthorhombic crystals grew mainly as long rods with a diamond-shaped cross-section. The space group of these crystals is $P2_1P2_1P2_1$ [8,9]. In this space group 3 two-fold screw axes are parallel to the a*,b*,c* directions. Crystal growth occurs preferentially in the direction of the a* axis, but the external faces of the crystal are not parallel to the a*b* or a*c* planes (Ducruix, A., Arnoux, B. and Reiss-Husson, F., unpublished). Thin crystals (less than 10 μm thickness) were selected for the spectroscopic investigations.

2.2. *Spectroscopic techniques*

Absorption and linear dichroism spectra of crystals were recorded on a single beam microspectrophotometer described in [15]. For absorption measurements, crystals were transferred from the mother liquor to a microcell formed by two cover slides separated by a spacer of 10 μm or 20 μm thickness. Due to the dimensions of the crystal plates (up to 300 × 100 × 20 μm), they were aligned parallel to the plane of the microcell. Spectra were recorded between 450 and 1000 nm with a resolution of 2 or 4 nm. The measuring light intensity was varied using neutral density filters in order to account for the actinic effects described below. Light-induced absorbance changes were recorded as described in [15]. All measurements were performed at room temperature.

3. RESULTS AND DISCUSSION

3.1. *Polarized light absorbance spectra*

Two different morphological forms were observed for crystals grown under the same conditions of crystallization, and often in the same capillary. The polarized absorbance spectra of crystals of both forms are shown in fig.1A and B. Both crystals were oriented in the sample holder to have their long axis in the 0° polarization direction. The crystal plane extends in the x-y plane as defined in the inset of fig.1A. A very high dichroism for the Q_y transitions of the BChl dimer, the monomeric BChls and the BPheos is observed for crystals growing as thin platelets (fig.1A). The Q_y transitions of the primary electron donor and of the BChl monomers are preferentially oriented in the y-direction, whereas the BPheo Q_y transition moments appear in the x-direction. With crystals grown in the form of hollow tubes, the dichroism of the BChl and BPheo Q_y transitions is less pronounced (fig.1B), and both bands appear broadened and flattened. Spectra from planar fragments of crushed 'tubes' correspond to those of thin platelets, supporting the assumption of two different morphological forms of the same crystal. A series of spectra at various polarizer angles of a tube crystal missing side walls is shown in fig.1C. The shift of the BPheo absorption from 762 nm

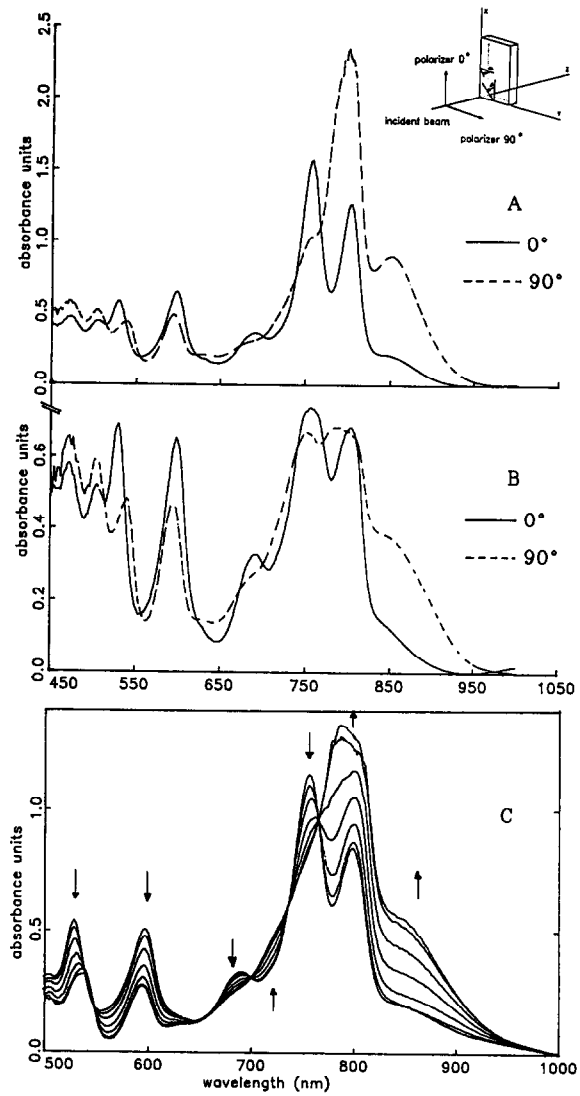

Fig.1. Polarized light absorbance spectra of *Rb. sphaeroides* Y single crystals grown in different morphological forms: (A) thin platelet; (B) hollow tube with rectangular cross-section; (C) tubular form missing side wall. Crystals were aligned with the long axis in the x-direction corresponding to 0° polarizer angle as indicated in the inset. (Full line: polarizer at 0°; dashed line: polarizer at 90°.) In (C), the direction of the polarizer was varied from 0° to 90° in steps of 15°. Arrows indicate sequence of spectra from 0° to 90°. Crystal sizes: up to 60 × 150 μm. Spectra were recorded at ambient temperature.

(0°) to 750 nm (90°) and for the monomeric BChl absorption from 800 nm (0°) to 790 nm (90°), in connection with stable isosbestic points, indicates the presence of two individual transitions for each BChl and BPheo. According to spectra of *Rb. sphaeroides* RCs reduced with sodium borohydride [16–19], one would assign the transition at 800 nm (0°) to the BChl monomer bound to the M subunit [20]. The two individual Q_y transitions of the BPheo can probably be assigned to the one associated with the M subunit (750 nm, 90°) and with the L subunit (762 nm, 0°), respectively, on the basis of photochemical trapping and equilibration experiments [21].

In the spectral region from 450 to 650 nm, the dichroism of the crystal spectra closely resemble each other. A preferential orientation of the Q_x transition in the 0° direction is observed, with only a small but significant shift of the BChl Q_x absorbance maxima (from 593 nm at 0° to 587 nm at 90°). However, a large shift of the absorbance maximum of the BPheo Q_x transition from 528 nm (0°) to 540 nm (90°) indicates a high orientation of the transition moments of the two pheophytins along the x- (BPheo M) and the y-axis (BPheo L). The dichroism of the carotenoid absorption at 469 nm and at 504 nm indicates its preferential orientation in the y-axis, i.e. with the projection parallel to the projection of the P Q_y transition. A much higher dichroism, however, would be expected if the carotenoid transition moment were fully oriented in the x-y plane. Spectra of tilted crystals will give more precise information on the spatial orientation of the carotenoid.

Comparing spectra of freshly grown crystals with those of crystals aged several weeks, a decrease in the primary donor absorption band was observed, concomitant with a decrease of the carotenoid absorption. In such cases, a dichroic 680 nm band of oxidized BChl was observed. Addition of ascorbate to the mother liquor fully restored the primary donor absorption, but had little or no effect on the carotenoid bands and in the 680 nm region. This instability of the carotenoid in the isolated RC is probably enhanced through the influence of the close-lying oxidized primary electron donor.

RC crystals have been shown to contain between ≈50% [22] and almost 100% [4,20] of the secondary electron acceptor Q_B. In spectroscopic investigations of crystals, however, a high content of Q_B can lead to an accumulation of reaction centers in the state $P^+Q_B^-$ through the action of even weak measuring light. A slight increase of the primary donor absorption was indeed observed when spectra of crystals with ascorbate added to accelerate the back-reaction were recorded, or when the measuring light intensity was reduced considerably. Either of these conditions was kept for the measurements.

3.2. *Dynamics of charge separation in crystallized reaction centers*

In order to induce charge separation in the crystallized RC, a laser beam coaxial with the measuring light was modulated with a chopper to obtain a period of illumination long enough to create a saturated charge-separated state (P^+Q^-) and a dark period long enough to allow charge recombination. Fig.2 shows kinetic traces recorded at 865 nm and at 1000 nm with the analyzing light at higher intensity than for the absorbance spectra. The absorbance decrease at 865 nm reflects the bleaching and recovery of the primary electron donor (P), whereas the absorbance increase at 1000 nm corresponds to the appearance and decay of the dimeric BChl a cation radical [23]. An analysis of the kinetic parameters of charge recombination reveals a predominant proportion (≈70%) of a fast phase ($t_{1/2} \approx 75$ ms) with a smaller contribution (≈30%) of a slow phase ($t_{1/2} \approx 260$ ms) at 865 nm. At 1000 nm, however, significantly different kinetic parameters are observed: a slow phase (90%, $t_{1/2} \approx 1.3$ s), with only small contributions from a fast phase ($t_{1/2} \approx$ 170 ms). The different ratio of the slow and the fast kinetic components for the two signals can be explained by the actinic effect of the measuring light at 865 nm mainly on those RCs containing Q_B as an electron acceptor. The signal at 865 nm thus only reflects a small portion of the RCs containing Q_B. With the measuring light tuned at 1000 nm, or at 865 nm, but strongly reduced in intensity (not shown), no actinic effect occurs and the ratio of the amplitudes of the slow and the fast decay components reflects the ratio of those RCs with Q_B present and the RCs that have lost Q_B during the isolation or crystallization procedure. This allows the Q_B content of the crystalline RC to be determined: the amplitude of the slow phase (≈90% of

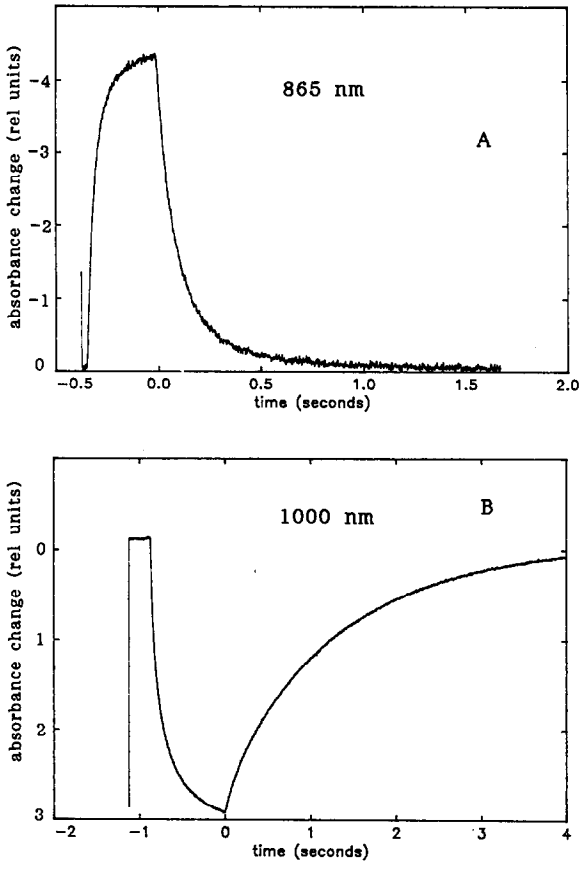

Fig.2. Transient light-induced absorbance changes at 865 nm (A) and at 1000 nm (B) of a single crystal of a *Rb. sphaeroides* Y RC. A laser beam (594 nm He-Ne line, polarized in the x-direction) coaxial with the measuring light was modulated with a chopper to create a saturated charge-separated (P^+Q^-) and a dark period (starting at $t = 0$). Conditions: average of 10 sweeps; measuring light polarized at 90°; crystal size approx. 50 × 100 μm; ambient temperature.

the total signal) observed at 1000 nm indicates that ≈90% of the RC in the crystal have kept Q_B.

The half-times of the charge recombination from Q_A and from Q_B measured at 865 nm and at 1000 nm differ considerably. We attribute this dependence on the measuring wavelength to different fractions of the RC in constant turnover through the action of the measuring light. The interaction of the reducing and oxidizing side of the RCs with their environment cannot be neglected in the crystal, where solvent and detergent comprise only about 70% of the total volume [24]. A similar influence of the concentration was also observed for hydrated films of RC (Mäntele, W., unpublished).

4. CONCLUSIONS

The spectroscopic characterization of crystals from the *Rb. sphaeroides* Y RC allows the identification of individual pigments through their absorption bands and the determination of the spatial orientation of the transition moments with respect to the morphologic crystal axes. In a previous study on *Rp. viridis* RC crystals [25], a consistent picture of spectroscopic and structural information was obtained. For the *Rb. sphaeroides* Y crystal, the projection of the carotenoid transition moment on the x-y plane is approximately parallel to that of the primary donor Q_y transition. Applying symmetry operations with the known arrangement of the reaction center in the unit cell, in connection with spectra of the tilted crystal, will thus allow the determination of the spatial orientation of the carotenoid.

Transient optical spectroscopy of crystals appears to be a useful probe for the functional properties of the RC. Light-induced charge separation in the crystallized RC has been found to proceed to the secondary acceptor quinone. Analysis of the rates of charge recombination allows the determination of the amount of Q_B present in >90% of the RCs. Moreover, investigation of the dynamics of the light-induced reactions indicates that the structure of the RC remains essentially unaltered upon crystallization.

Acknowledgements: The authors would like to thank Mrs Danièle Clérot for expert assistance in RC purification, Dr A. Ducruix for helpful discussions as well as P. Gutgsell and A. Becker for their help in data handling and analysis.

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