EPR detected triplet formation in a single crystal of reaction center protein from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26

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Single crystals were obtained from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* R-26. At low temperatures these crystals are highly photoactive, as is demonstrated by the observation of the T_0-populated donor triplet state. The angle dependence of the transitions of this triplet state showed that the space group of this crystal is orthorhombic.

Bacterial photosynthesis Rhodopseudomonas sphaeroides *R-26* *EPR* *Reaction center triplet* *Protein crystal*

1. INTRODUCTION

A direct and very sensitive method to obtain information about the photoactivity and the orientation of the primary donor in single crystals from the reaction center protein from the photosynthetic bacterium *Rhodopseudomonas viridis* [1] has been found in the EPR technique. Authors in [2] showed that the triplet state of the primary donor, P^T_{990}, could easily be measured with EPR in a single crystal from reaction center protein from *R. viridis*. Due to the inherently strong anisotropy associated with the EPR spectrum of the triplet state and the large electron spin polarization in the three triplet sublevels, angles were obtained with an accuracy of $\pm 1°$. Highly polarized, spin sublevels [3], in which the middle triplet sublevel contains all of the initial population of triplet, are indications of triplet formation via the radical-pair mechanism [4]. In fact, only in photosynthetic systems has this specific mechanism for triplet polarization in organic molecules been observed. For a review on the radical pair mechanism in photosynthesis see [5].

Measurements of the anisotropy associated with the EPR triplet resonance as a function of applied magnetic field gives the orientation of the donor special pair as probed by the excited donor triplet state relative to the external crystal surfaces. Since the radical pair mechanism and its resulting polarized triplet strongly depend on very specific radical pair interactions and radical pair lifetimes, the observation of this highly polarized middle triplet sublevel in the crystal not only demonstrates the photoactivity of the crystal, but it also shows that the photoreaction in this crystal is essentially identical to the in vivo photoreaction. Combining triplet data with the orientation of P^+I^- and Q^- should determine the organization of the photoactive part of the reaction center protein.

We report here on investigations on the angle dependence of the triplet state of the primary donor in a new single crystal of *R. sphaeroides* R-26. Although the volume of the crystal was typically less than 5 nl, P^T_{870} could easily be observed by low temperature EPR. These measurements show for the first time the high photoactivity of these crystals at low temperature. The angular dependencies of the triplet anisotropy are simulated and explained with an orthorhombic space group. This is in contrast to the P2 space group for a crystal reported earlier [6].

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2. MATERIALS AND METHODS

Reaction center proteins from *R. sphaeroides* R-26 were obtained as in [7]. The reaction centers were dialyzed against 1% *n*-octyl-β-D-glucopyranoside, 10 mM Tris (pH 8) followed by concentration to $A^{800} = 40$ using ultrafiltration. Crystals were obtained using essentially the method in [8]. Briefly, a solution of 8% polyethyleneglycol (PEG 6000 or PEG 4000), 0.25 M NaCl, 0.8% *n*-octyl-β-D-glucopyranoside, 2 mg/ml reaction center protein, 10 mM Tris (pH 8) was put to equalize with 25% PEG (4000 or 6000, respectively) and 0.8 M NaCl at room temperature in the dark. After about 2 weeks crystals were observed; after about 6 weeks the crystallization process was complete. Under crossed polarized light the normally blue-colored crystals were bright orange-red. When a crystal was taken out of the liquid and put into a solution of 0.1% lauryl dimethylamine oxide, 10 mM Tris (pH 8), 1 mM EDTA, it dissolved quickly. When several crystals were dissolved, it was possible to measure the absorption spectrum which was the same as a reaction center solution. Examination under a microscope showed that the overall dimensions of the crystal were about $1 \times 0.03 \times 0.1$ mm (these dimensions will be referred to as the *z*-, *y*-, and *x*-, respectively, of an orthogonal axis system).

A crystal used for EPR measurement was first carefully transferred into a solution containing 1.5 M sucrose, 25% PEG (4000 or 6000, respectively), 2% *n*-octyl-β-D-glucopyranoside, 0.5 M NaCl, 1 M sodium ascorbate, 30 mM Tris (pH 8). The crystal was then positioned in the desired orientation on a quartz rod and frozen in the dark or under illumination. As with the crystals from *R. viridis*, the solution containing 1.5 M sucrose and 25% PEG gave a clear glass at low temperatures and no damage due to freezing and thawing was observed.

Light modulation and the EPR spectrometer was as in [4]. PEG was purchased from Fluka, *n*-octyl-β-D-glucopyranoside was obtained from Sigma.

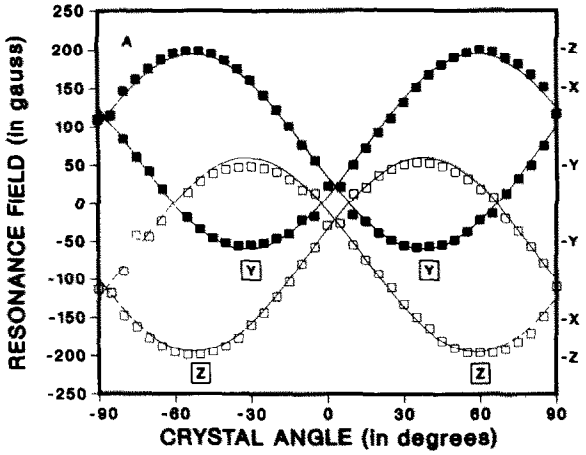
3. RESULTS

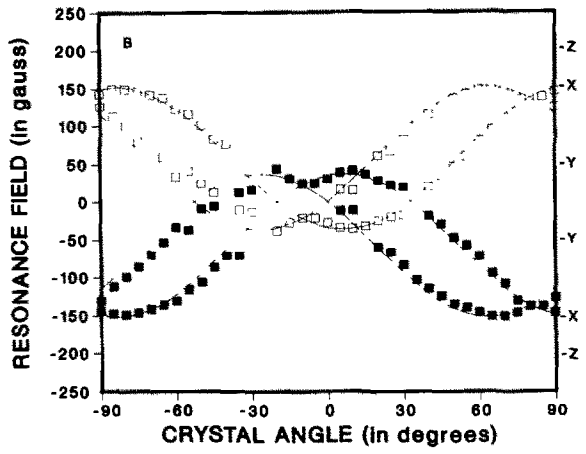
No difference was found in the amplitude of the light-indued EPR spectrum of P^{T}_{870} whether the crystals were frozen in the dark or under illumination. Experiments with a solution of reaction center protein of *R. sphaeroides* R-26 showed that when the non-crystalline reaction centers were in the solution used for the crystals, Q was already reduced prior to freezing.

To determine the position of the triplet molecular axes in a single crystal of reaction center protein from *R. viridis*, it was sufficient to rotate the crystal around two of its crystal axes, assuming the zero field splitting parameters of P^{T}_{990}. This was due to the symmetry inside the crystal brought upon by the $P4_12_12$ space group. In a single crystal from reaction center protein of *R. sphaeroides* R-26, however, we found that it was necessary to rotate the crystal around all 3 of the crystal axes to obtain full information about the position of the triplet axes in the crystal. Fig.1 shows the results. In this figure, the points representing the magnetic field at which absorption or emission occurs are shown as a function of the angle between the crystal axes and the magnetic field. The minimum linewidth of a transition peak was found to be 10.3 G.

As can be seen from fig.1, the maximum number of lines observed is 4 and the minimum is 2. Interestingly, more than 4 lines in the spectrum of P^{T}_{870} were found when the crystal was not exactly aligned, i.e., when there was no crystal axis perpendicular to the magnetic field. This effect was very drastic and a misalignment of about 10° was already enough to introduce more than 4 lines. Also, the appearance of these extra lines resulted in severe line broadening, which produced drastically decreased amplitudes.

The findings in fig.1 were simulated by computer (solid line) with the assumption that the space group of the crystal was orthorhombic and assuming the zero field splittings of P^{T}_{870}, obtained from a solution of reaction centers. With this simulation, the following angles between the triplet axes (x_T,y_T,z_T) and the crystal axes (x,y,z) were as follows: x_T with x, 19°; x_T with y, 107°; x_T with z, 82°; y_T with x, 87°; y_T with y, 54°; y_T with z, 36°; z_T with x, 109°; z_T with y, 139°; z_T with z, 55° (the error in these angles is 2–4°). The other three orientations of the triplet axes in the crystal are determined by the orthorhombic space group; they are found by the three following transformations: $x \longrightarrow -x$, $y \longrightarrow -y$; $x \longrightarrow -x$, $z \longrightarrow -z$; $y \longrightarrow$





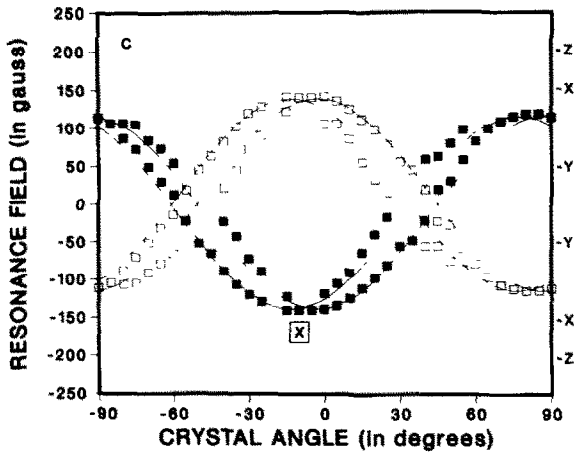


Fig.1. The four triplet EPR resonance positions as a function of magnetic field H_0 for three different rotations around H_0. (A) Rotation around the x-axis of the crystal; (B) rotation around the y-axis; (C) rotation around the z-axis (see section 2 for definition of the axes). Open squares indicate enhanced absorption, closed squares emission. x, y and z in the right-hand figure axis are the resonance positions if the magnetic field H_0 were along the triplet x, y and z axes. These transitions were obtained from a solution of reaction center protein from *R. sphaeroides* R-26. x , y and z indicate the positions where the magnetic field is aligned (or almost aligned) with one of the three triplet axes in the crystal. Solid lines represent the computer simulations assuming an orthorhombic space group. Temperature, 5 K; microwave power, 1 mW; modulation amplitude, 16 G; light modulation frequency, 1 kHz.

$-y, z \longrightarrow -z$. Computer simulation, again assuming an orthorhombic space group, also demonstrated that a misalignment of only 5° increased the number of triplet transitions to a maximum of 8. Simulating these effects with a P2 space group always resulted in a maximum of 4 resolved, triplet EPR transitions.

4. DISCUSSION

The typical sample volume used in the R-26 single crystal experiment is 3 nl or 30000 times less volume than in the typical non-crystalline R-26 experiment for only slightly smaller signals. If the crystal's internal order were to be randomized, a 3 nl sample volume of reaction centers would be undetectable by standard EPR. If the crystals were composed of several crystals or contained fractured or splintered single crystals, only complicated spectra, if any, could be observed.

The large amplitude of the triplet signals from the crystal, with a volume less than 5 nl, is a direct indication of the strong electron spin polarization (ESP) of the triplet, as is expected for P^T_{870}. In fact, it can be seen from fig.1 that the ESP has the familiar $A_{z_I}E_{x_{II}}E_{y_{II}}$ $A_{y_I}A_{x_I}E_{z_{II}}$ (E, emission; A, enhanced absorption) spin polarization pattern. This is best seen where the crystal is in such an orientation that the magnetic field is aligned with the x_T, y_T or z_T direction of the triplet axis. This is indicated in the figure. Another indication for the origin of this triplet spectrum is that the spectra could be simulated using the D and E values (D =

202 G and $E = 34$ G) from a solution of RC's from R-26.

With the observation of P^T_{870}, we demonstrated the high photoactivity at low temperatures of a single crystal from *R. sphaeroides* R-26. The light-induced appearance of P^T_{870} demonstrates more than the photoactivity of this crystal. Since the formation of this radical pair populated triplet state depends strongly on specific interactions in the most primary radical pair, $P^+_{870}I^-$, and on their decay rates, it shows that these crystals exhibit identical photoreactions to the protein in solution and the in vivo bacterium. The fact that P^T_{870} is even observed when the crystal is frozen in the dark is thought not to be an indication of the non-activity of Q at low temperature. Preliminary results indicate that Q is prereduced prior to freezing, most likely due to the high concentration of sucrose and PEG. Further evidence is that the reversible light-induced EPR spectrum of P^+_{870} can be observed at 5 K when sodium ascorbate is not added and the crystal is frozen in the dark.

The results presented in fig.1 could be simulated very well assuming an orthorhombic space group for the crystal (solid line, fig.1). It could, however, also be simulated with a P2 space group. Magnetically there are as many different orientations in a P2 space group as in an orthorhombic space group when one is along one of the symmetry axes. But a P2 space group would always give a maximum of 4 lines, at various orientations of the crystal relative to the magnetic field. However, a slight misalignment of the crystal increased the number of experimental lines to at least 6 (and probably 8 at which point the lines are not resolved anymore). Spectra of such a misaligned crystal cannot be simulated by a P2 space group. This was only possible with an orthorhombic space group. We therefore feel strongly that the space group of this type of crystal is orthorhombic. This is in contrast to what has been measured by authors in [6], who found a P2 space group for a single crystal prepared in a similar manner, using X-ray spectroscopy. Preliminary X-ray data [9] indicate that the dimensions of the unit cell are different to those reported in [10]. One difference between the two crystallization processes is that in this case the reaction center protein was extracted with lauryl dimethylamine oxide followed by dialysing against *n*-octyl-*β*-D-glucopyranoside, while authors in [6] extracted the reaction center protein with *n*-octyl-*β*-D-glucopyranoside as detergent.

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REFERENCES

[1] Michel, H. (1982) J. Mol. Biol. 158, 562–567.
[2] Gast, P., Wasielewski, M.R., Schiffer, M. and Norris, J.R. (1983) Nature 305, 451–452.
[3] Dutton, P.L., Leigh, J.S. and Seibert, M. (1972) Biochem. Biophys. Res. Commun. 46, 406–413.
[4] Thurnauer, M.C., Katz, J.J. and Norris, J.R. (1975) Proc. Natl. Acad. Sci. USA 72, 3270–3274.
[5] Hoff, A.J. (1984) Q. Rev. Biophys., in press.
[6] Allen, J. and Feher, G. (1984) Bioph. J. Soc. abs. W-AM-A5.
[7] Wraight, C.A. (1979) Biochim. Biophys. Acta 548, 309–327.
[8] Garavito, R.M. and Rosenbush, J.P. (1980) J. Cell. Biol. 86, 327–329.
[9] Schiffer, M., personal communication.
[10] Allen, J.P., Isaacson, R.A., McPherson, A. and Feher, G. (1984) Biophys. J. Soc. abs., W-AM-A6.