BRIDGING THE GAP BETWEEN NATURAL AND ARTIFICIAL PHOTOSYNTHESIS*

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Summary

Considerable effort has been expended to develop artificial models that mimic various aspects of natural photosynthesis. The most direct and complete bridge between natural and artificial systems would be provided by the detailed structure of a functioning photosynthetic reaction center protein complex. When sufficient structural knowledge is available, a better understanding of the dynamics and functions occurring in the primary act of photosynthesis will be possible in a straightforward manner, particularly with the recent development of single crystals of reaction center proteins derived from photosynthetic bacteria. Consequently, single crystals of protein reaction centers from photosynthetic bacteria currently serve as the best fiduciary system for the development of model systems that use light energy to produce charge separation.

The central feature of natural photosynthesis is the utilization of several very rapid, and slightly exothermic, electron transfer reactions following the creation of an excited singlet state in the chlorophyll chromophores of a reaction center protein complex. With regard to this aspect of the natural process, the development of model systems based on photodriven multistep electron transfer reactions is progressing rapidly.

In this paper we establish the following five major points.

(1) We report a new *Rhodopseudomonas sphaeroides* R-26 reaction center single crystal.

(2) We demonstrate that these crystals function in a manner similar to the intact organism.

(3) We develop a probe of dynamics and structure based on the triplet state that works in single crystals, in liquid or solid solutions of reaction centers, in chromatophores or in intact organisms.

(4) At the same time the triplet state provides an internal "goniometer" for establishing a structural baseline for spectroscopic studies relevant to X-ray studies.

(5) Finally, we prove that it is possible to study these crystals over a large temperature range (5 - 300 K) without crystal damage or loss of internal order.

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This final point is particularly significant since a large temperature range for various experimental observations is required in order to determine such things as the activation energies of the various reaction steps involved in the dynamics. These temperature studies will be necessary to establish the validity of existing electron transfer theory in the understanding of natural and artificial photosynthesis.

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1. Introduction

A significant expenditure of work has been devoted to the development of artificial models that mimic various aspects of natural photosynthesis. It has been assumed in such endeavors that the process of natural photosynthesis is understood to an extent which is sufficient for the building of realistic biomimetic systems to be possible. As yet, however, the process of charge separation is not fully understood in natural photosynthesis and consequently model systems are also limited in their ability to duplicate the natural process. We suggest that the most direct and complete bridge between natural and artificial photosynthesis would be provided by the detailed structure of a functioning photosynthetic reaction center protein complex. The more detailed the structural information is, the more rapid will be the advance toward a better understanding of the dynamics and functions occurring in the primary acts of photosynthesis and artificial photosynthesis.

These qualifications are not intended to imply that little is known about photosynthesis since in actuality a great deal is understood about this complex process. Nevertheless, very little of the structural details of the process of primary charge separation in natural systems has ever been established, at least not in decidedly precise terms. Soon, however, the situation promises to change with the recent development of single crystals of reaction center proteins derived from photosynthetic bacteria [1 - 4]. As a direct consequence, protein reaction centers from photosynthetic bacteria currently serve as the best fiduciary system for the development of model systems that use light energy to produce charge separation.

This charge separation process leads to the creation of chemical reducing and oxidizing power. In purple bacteria the source of the reducing electrons is provided by a sacrificial electron donor such as an organic substrate H_2A. Likewise, model systems based on photosynthetic systems also rely on sacrificial electron donors for prolonged charge separation. Of considerable importance is the fact that green oxygen-evolving plants ultimately use H_2O, the ideal sacrificial electron donor, for the two photoreactions that comprise the so-called Z scheme. The structural aspect of the understanding of photosynthesis requires the complete protein structural determination by X-ray diffraction methods. The X-ray structure is by far the most significant means of bridging the gap between natural and artificial photosynthesis.

However, investigation by X-rays alone is not sufficient because as yet the technique provides no dynamic information directly and thus is not suited for probing the functions of the various components and electron transfer steps that constitute the process of photosynthesis. In addition, the X-ray technique can be applied only to single crystals and would be of little value in studying photosynthesis *in vivo*. Consequently, only by supplementing the X-ray structure with data provided by other more versatile forms of spectroscopy such as transient optical spectroscopy and electron paramagnetic resonance (EPR) spectroscopy will the functions and dynamics of photosynthetic charge separation be established. Any new functions that become clear because of the availability of the X-ray structure will probably result from the combining of X-ray data with EPR and/or optical spectroscopy. In this way the X-ray structure will influence greatly the study of the dynamics of photosynthesis ultimately leading to the development of accurate models of artificial photosynthesis.

In this paper we establish the following five major points.

(1) We report a new reaction center single crystal from the bacterium *Rhodopseudomonas sphaeroides* R-26.

(2) We demonstrate that these crystals function in a manner similar to the intact organism. Of course, for the purposes of modeling fast photosynthetic reactions, workers need not be concerned with whether or not the process in these crystals is identical with that of the intrinsic natural system; for model building it is sufficient that very fast and efficient photochemistry occurs that has only some of the features of the natural process.

(3) We develop a probe of dynamics and structure that works in single crystals, in liquid or solid solutions of reaction centers, in chromatophores or in intact organisms. Because of the high speed of the photosynthetic process and the small size of the protein crystals (volume, approximately 3 nl), this appears at first glance to be an impossible task. However, in our previous work we have established that the triplet state that occurs in photosynthetic systems is a unique probe of picosecond charge separation.

(4) At the same time the triplet state provides an internal "goniometer" for establishing a structural baseline for spectroscopic studies relevant to X-ray studies. We conclude that high symmetry is likely for the reaction center complex which in purple bacteria involves a special pair primary donor [5].

(5) Finally, we prove that it is possible to study these crystals over a large temperature range (5 - 300 K) without crystal damage or loss of internal order. This final point is particularly significant since a large temperature range for various experimental observations is required in order to determine such things as the activation energies of the various reaction steps involved in the dynamics. These temperature studies will be necessary to establish the validity of existing electron transfer theory in the understanding of photosynthesis.

2. Materials and methods

Reaction center proteins from *R. sphaeroides* R-26 were obtained using the method from ref. 6. We have prepared two types of reaction center crystal.

(1) Following the method of Michel [1] we have produced large (approximately 2 mm × 0.6 mm × 0.8 mm) single crystals of *R. viridis*. The work of Michel [1] was a major breakthrough and the X-ray structure will soon be available from the efforts of Deisenhoffer *et al.* [7].

(2) Following the methods of Garivito [4, 8], we have grown single crystals of *R. sphaeroides* R-26 (about 1 mm × 0.1 mm × 0.03 mm in size). These crystals have a structure different from the crystals described using the X-ray data of ref. 2. Preliminary X-ray data show that the unit cell size of our crystals will be smaller than that of *R. viridis* [9]. Hopefully, the absence of the cytochromes will make this simpler than the situation in crystals of *R. viridis*. The only known disadvantage of this crystal is its small size.

Under crossed polarized light the crystals were bright orange–red. The crystals were soluble in a solution of 0.1% LDAO, 10 mM tris(hydroxymethyl)aminomethane (Tris) at pH 8 and 1 mM ethylenediaminetetraacetic acid and gave an absorption spectrum which was the same as that of a standard reaction center solution obtained without the single-crystal preparation step. One crystal at a time was used for the electron spin resonance (ESR) measurement. Each crystal was transferred via solution (1.5 M sucrose; 25% poly(ethylene glycol) (4000 or 6000); 2% *n*-octyl-β-D-glucopyranoside; 0.5 M NaCl; 1 M sodium ascorbate; 30 mM Tris at pH 8) to a quartz rod, positioned in the desired orientation and frozen in the dark or under illumination. The crystals in this sucrose solution gave a clear glass at low temperatures and no damage with freezing and thawing was observed. Light-modulation-detected EPR was employed [10].

3. Results

To determine the position of the primary donor molecular axes via the anisotropic triplet state zero field interactions in a single crystal of reaction center protein from *R. viridis* it was sufficient to rotate the crystal around two of its crystal axes because of the relationship of symmetry between the P_{241} space group and the donor axes. In a single crystal from the R-26 reaction center protein, however, we found it necessary to rotate the crystal around all three crystal axes to obtain full information about the position of the triplet axes (*i.e.* donor axes) in the crystal.

Figure 1 shows the experimental spectroscopic results together with calculations based on one set of Euler angles and the triplet zero field parameters (D = 167 G; E = 34 G). In general, the magnetic field at which absorption or emission occurs is shown as a function of the angle between the crystal axis and the magnetic field. The minimum linewidth of a transition peak was found to be 10.3 G in R-26 (Table 1).

As can be seen in Fig. 1, the observed maximum number of EPR transitions per magnetic field value is four and the minimum is two. Importantly, more than four lines in the spectrum of the primary bacteriopheophytin acceptor (P) were found when the crystal was not exactly aligned, *i.e.* when there was no crystal symmetry axis perpendicular to the magnetic field. This effect was very drastic and a misalignment of 5° - 10° was already enough to introduce more than four lines. Also, the appearance of these extra lines gave considerable line broadening, which results in drastically decreased amplitudes.

The changes in splittings and patterns of the EPR transitions as a function of crystal orientation in the laboratory magnetic field can be

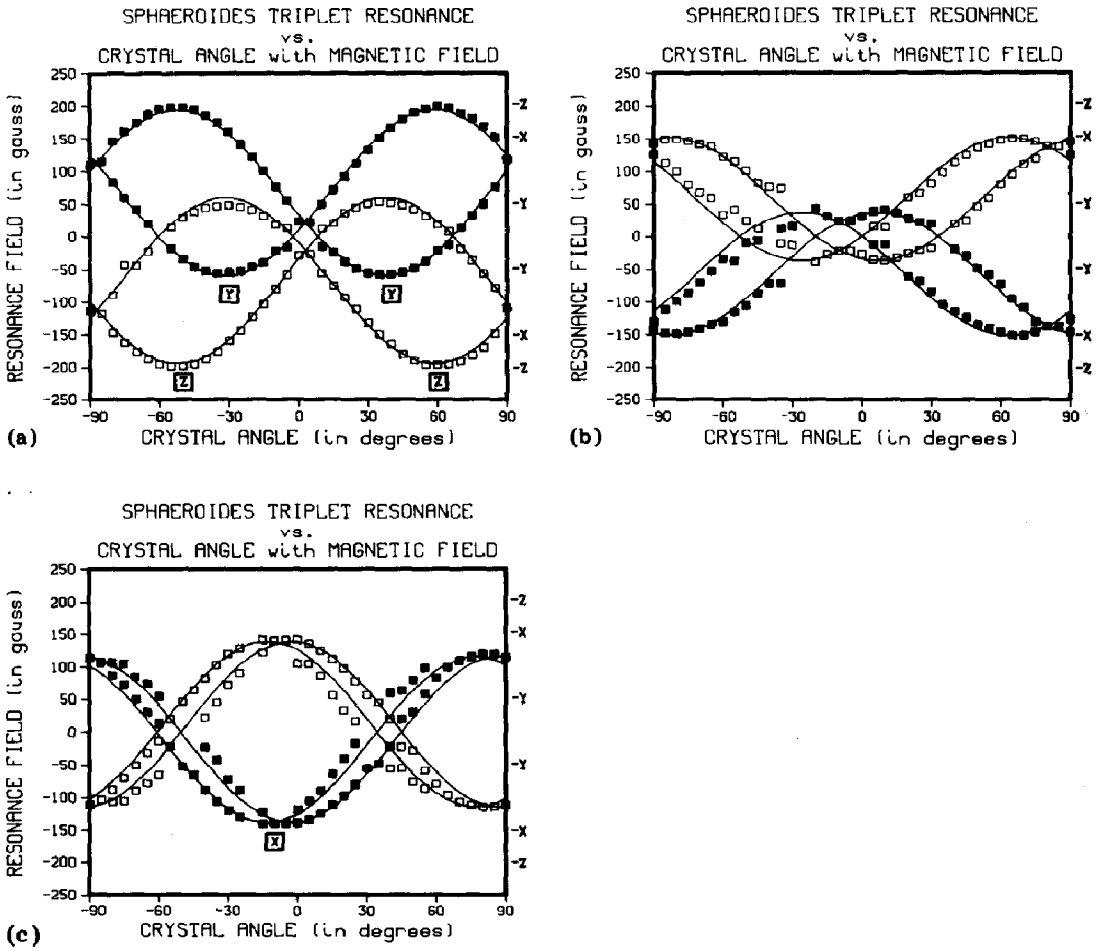

Fig. 1. The four triplet ESR positions as a function of magnetic field H_0 for three rotations with the relevant crystal axis perpendicular to H_0 (temperature, 5 K; microwave power, 1 mW; modulation amplitude, 16 G; light-modulated frequency, 1 kHz): (a) rotation around the long axis of the crystal (□, Z emission; ■, Z absorption); (b) rotation around the short axis of the crystal (□, Y emission; ■, Y absorption); (c) rotation around the medium-length axis of the crystal (□, X emission; ■, X absorption).

TABLE 1

Triplet *versus* cation electron paramagnetic resonance linewidths

Organism	*Hydrogen species*	*EPR linewidth* (G)	
		Triplet	*Cation*
R. viridis	^1H	11.3	11.5
R. viridis	^2H	6.8	4.6
R. sphaeroides R-26	^1H	10.3	9.6

TABLE 2

Euler angles locating the primary donor in R-26 single crystals

R_{z1}	$+10° \pm 2°$
R_{x2}	$+55° \pm 2°$
R_{z3}	$-23° \pm 2°$

reproduced with calculated triplet EPR spectra (Fig. 1, solid lines) by assuming a unit cell containing three mutually perpendicular twofold rotation axes for the triplet donor, resulting in a minimum of four proteins or donors per unit cell. This simulation process also provides the relative orientation of the donor P870 units within the crystal unit cell. The Euler angles (positive angle is a clockwise rotation as one faces the positive axis) are in Table 2. Computer simulation also demonstrated that a misalignment of only 5° - 10° increased the number of triplet transitions to a maximum of eight. The simulation of these effects with a single twofold axis space group always resulted in a maximum of four lines.

4. Discussion of results

The typical sample volume used in the R-26 single crystal experiment is 3 nl or 30 000 times less volume than in the typical non-crystalline R-26 experiment for only slightly smaller signals. If the internal order of the crystal were to be randomized, a 3 nl sample volume of reaction centers would be undetectable by standard EPR. If the crystal were composed of several crystals or contained fractured or splintered single crystals, only complicated spectra, if any, could be observed. Since simple and intense triplet state spectra were observed, this demonstrates that it is possible to study these crystals from 5 to 300 K with no apparent damage.

The highly intense EPR signal in single crystals is relevant to the question of charge separation and model systems. It has been shown that the triplet state in photosynthetic preparations arises during the annihilation of

light-induced picosecond charge separation through the back reaction to the initial ground state of the system, *i.e.* [10]

$$P870^{+}\ B\ P^{-} \longrightarrow {}^{*}P870^{3}\ B\ P \longrightarrow P870\ B\ P$$

where P870 is the special pair primary donor, P is the primary bacteriopheophytin acceptor and B is the bacteriochlorophyll B800, the intermediate acceptor. This reverse charge separation chemistry occurs when the quinone moieties that are located further in the reaction chain are not able to function as electron acceptors. The triplet state EPR signal that arises from this charge separation process is quite intense because of large deviations from Boltzmann population within the triplet sublevels. In fact, only the middle triplet level $|0\rangle$ is initially populated to any significant extent. This is a result of the so-called radical pair mechanism [10]. It is especially important to note that this charge separation collapse gives rise to the triplet state EPR signal and that the EPR transitions always originate from the overpopulation of $|0\rangle$ compared with $|+\rangle$ or $|-\rangle$, regardless of the angle that the external magnetic field makes relative to the donor orientation. When triplet states are formed without this charge separation, *i.e.* without the radical pair mechanism, $|0\rangle$ will be underpopulated relative to $|+\rangle$ or $|-\rangle$ for at least some orientations of the donor relative to the external magnetic field [10]. Since the EPR transition intensity is proportional to the population differences, signals originating from triplets produced by the radical pair mechanism are quite intense.

The overpopulation of $|0\rangle$ regardless of the orientation of the external magnetic field has as yet only been observed using triplet EPR spectroscopy in photosynthetic preparations and in some simple non-organic solid state systems but not in model photosynthetic systems. From liquid solution studies of model charge separation, such a reaction scheme is known to occur. However, once these systems are frozen, which is necessary in order to perform triplet EPR spectroscopy, according to EPR the systems no longer function with this mechanism to any measurable extent.

In both crystal systems, *R. sphaeroides* and *R. viridis*, we have observed that only the middle triplet sublevel is significantly overpopulated and remains so for all angles that the crystal makes with the external magnetic field. This proves the photoactivity of these single crystals at low temperatures. At the same time it illustrates that normal redox chemistry proceeds rapidly and reversibly in these single crystals.

Moreover, this EPR triplet signal polarization (*i.e.* this $|0\rangle$ sublevel overpopulation) provides a working criterion for artificial photosynthesis. If such a mechanism were in operation in a model system, it would strongly suggest the four following points.

(1) Picosecond charge separation occurred from the singlet state.

(2) Charge separation lasted for tens of nanoseconds.

(3) The energy of the charge separation state is above the energy of the triplet state of the primary donor.

(4) The process is temperature independent and can occur at low temperatures (5 K) in the solid state.

This fourth property is one of the most significant aspects of natural photosynthetic charge separation, namely charge separation occurs from 350 to 5 K. This seems to indicate that the protein either provides a low dielectric medium for charge separation or plays a special role in neutralizing the electrostatics of charge separation.

For the purposes of additional interpretation of these single-crystal data we assume, as has been previously shown, that the triplet state resides in the primary donor P870 and that the reaction center proteins do not aggregate (*e.g.* dimerize) in a manner such that the asymmetric unit has a twofold triplet symmetry axis parallel with a symmetry axis of the whole crystal. Also, we assume that any local symmetry axes of the chromophores within a single reaction center protein are not coincident with the external crystal symmetry axes. The simplest EPR spectrum consists of a single pair of EPR transitions indicating that all donor molecules are magnetically equivalent within the resolution of the EPR transition linewidth. A pair of triplet lines is expected for a single magnetic site representing $|0\rangle$ to $|-1\rangle$ emission and $|0\rangle$ to $|+1\rangle$ enhanced absorption.

Given the above assumptions, the experimental observation of a single magnetic site indicates a single P870 donor molecule per reaction center. Because other work has shown that a protein reaction center contains four bacteriochlorophyll molecules and that the oscillator strength of the spectra of two bacteriochlorophyll molecules disappears with the formation of this triplet state, the triplet state resides in a dimer. If two monomeric triplet states in each asymmetric unit caused the reduction in the oscillator strength by the appropriate amount, more than one single magnetic site would be observed.

In addition, the triplet zero field splittings of the reaction centers are considerably reduced from that of the corresponding monomeric chlorophyll, especially in *R. viridis* (Table 3). This was first noted by Dutton *et al.* [11] who correctly interpreted this change in zero field properties as consistent with the special pair model for the donor cation.

Because the triplet EPR linewidth is reduced significantly in value on complete deuteration of the reaction center protein complex, electron–

TABLE 3

Zero field splitting parameters of bacteriochlorophyll *versus R. viridis*

System	*D value* (G)	*E value* (G)
Bacteriochlorophyll b	228	59
R. viridis	167	34

D(bacteriochlorophyll b)/D(*R. viridis*) = 1.37

nuclear hyperfine interactions dominate the triplet linewidth (Table 1). As can be seen from Table 1 the value of the ^{1}H *R. viridis* or ^{1}H *R. sphaeroides* triplet linewidth is almost identical with that of the corresponding ^{1}H *R. viridis* or ^{1}H *R. sphaeroides* cation linewidth observed for $P890^+/P870^+$. We have just concluded on the basis of zero field splitting considerations that the triplet state resides in a dimer. Since the similarity in triplet *versus* cation linewidth is required by the special pair model, we also conclude that the linewidth data of Table 1 support the special pair model for the cation of the primary donor in both *R. viridis* and *R. sphaeroides*.

Comparison of triplet linewidth with cation linewidth is possible because we have single crystals and since the triplet state can be most simply considered to be a cation and an anion simultaneously on the same molecule. It is interesting to note that the ^{2}H *R. viridis* triplet linewidth is noticeably larger than the ^{2}H cation linewidth. This is to be expected since Fajer and coworkers [12] have shown that in anions the spin density of the unpaired electron increases on the nitrogen atoms in comparison with the cation. In the fully deuterated systems the HFI of the nitrogen atoms dominates the anion linewidths. Since the triplet is the average of the cation and the anion, we expect the triplet linewidth to be greater than the cation and less than the anion.

Thus we conclude that both the triplet and the donor cation reside in a special pair of molecules. Such delocalization for two greatly different electronic states (the cation is charged whereas the triplet is neutral) suggests that the special pair and its immediate surroundings have C_2 symmetry. This is because in artificial photosynthesis studies the slightest asymmetry introduced into a model of the special pair causes the localization of the cation or triplet on a single member of the pair. The asymmetry typically arises from the solvent or local environment. These model studies suggest that in order to support the concept of a special pair the immediate environment of the natural donor must be symmetrical. That appears to be possible only with a dual pathway for electron flow. Since the immediate environment of P860 probably involves the intermediate acceptor bacteriochlorophyll (B800 in *R. sphaeroides*) and the primary bacteriopheophytin acceptor, we propose a dual pathway for electron flow, *i.e.* two electron acceptor networks. This is possible since the reaction center has two B800 molecules and two pheophytin molecules. Likewise this suggests two or four quinone molecules and two iron atoms at the secondary electron acceptor site.

Thus we have shown that the photoexcited triplet state is an especially convenient probe for picosecond-induced charge separation for *in vivo* photosynthesis and should be employed as a major criterion for evaluating model charge separation. The triplet state can even provide information about the cation of the donor state. In this work we have also shown that in *R. viridis* and in *R. sphaeroides* the cation of the primary donor and triplet of the primary donor reside in a special pair of bacteriochlorophyll molecules and the likelihood of a dual pathway for electron transfer must be considered both in natural and in artificial photosynthesis.

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