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Magnitude and Direction of the Change in Dipole Moment Associated with Excitation of the Primary Electron Donor in *Rhodopseudomonas sphaeroides* Reaction Centers†

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ABSTRACT: The magnitude and direction of the change in dipole moment, $\Delta\mu$, associated with the Q_y transition of the dimeric primary electron donor (special pair or P870) in *Rhodopseudomonas sphaeroides* reaction centers have been measured by Stark spectroscopy at 20 °C. The magnitude of $\Delta\mu$ is found to be $f^{-1}(10.3 \pm 0.7)$ D, where f is a correction factor for the local dielectric properties of the protein matrix. With the spherical cavity approximation and an effective local dielectric constant of 2, $f = 1.2$, and $|\Delta\mu|$ is 8.6 ± 0.6 D. $|\Delta\mu|$ for the Q_y transition of the special pair is approximately a factor of 3.4 and 2 greater than for the monomeric bacteriochlorophylls and bacteriopheophytins, respectively, in the reaction center. The angle, ζ, between $\Delta\mu$ and the transition dipole moment for excitation of the first singlet electron state of the special pair was found to be 24 ± 2°. The measured values of $|\Delta\mu|$ and ζ are combined to suggest a physical model in which the lowest excited singlet state of the special pair has substantial charge-transfer character and where charge is separated between the two monomers comprising the dimeric special pair. This leads to the hypothesis that the first charge-separated state in bacterial photosynthesis is formed directly upon photoexcitation. These data provide stringent values for comparison with theoretical calculations of the electronic structure of the chromophores in the reaction center.

Charge separation in photosynthesis is initiated by photoexcitation of the primary electron donor. In bacterial reaction centers (RCs)[1] the donor is a dimer of bacteriochlorophylls often called the special pair. The precise structure of the special pair in *Rhodopseudomonas viridis* RCs (P960) has been elucidated by X-ray crystallography (Deisenhofer et al., 1984). X-ray crystallographic studies of RCs from the species *Rhodopseudomonas sphaeroides*, whose special pair has an absorption maximum at approximately 870 nm (P870), are in progress. Preliminary evidence suggests that the gross features of the structures from the two bacteria are very similar (Chang et al., 1986; Allen et al., 1986), and we will adopt the *R. viridis* structure[2] as a working model for *R. sphaeroides*.

The electronic structure of the excited states of the special pair has been the subject of intense interest. Recent photochemical hole-burning studies from our laboratory (Boxer et al., 1986a,b) and related studies in Gröningen (Meech et al., 1985) led us to suggest that photoexcitation of the special pair produces a state with a substantially different equilibrium nuclear configuration than the ground state. We proposed that a state with substantial charge-transfer character could be important. This theme has been discussed in other analyses, notably some recent efforts to calculate the RC electronic absorption and CD spectra (Parson et al., 1985). A qualitative, but highly suggestive, abstract appeared several years ago, suggesting a substantial effect of an applied electric field (Stark effect) on the absorption spectrum of *R. sphaeroides* RCs (DeLeeuv et al., 1982). Stimulated by that work and the hole-burning studies, we have undertaken a quantitative investigation of the Stark spectrum of *R. sphaeroides* RCs, as this offers a direct approach to addressing the question of the degree of charge transfer associated with excitation of the special pair.

Application of an electric field can cause a number of changes in a sample: (i) if the molecules have a permanent dipole moment in the ground state, they will be aligned by the field; (ii) if the ground- and excited-state dipole moments are different, the excitation energy of the molecules will be increased or decreased depending on their orientation in the field; (iii) the difference in polarizability between the ground and excited state changes the energy of electronic transitions in a field; (iv) the magnitude of the transition dipole moment can change; (v) the electric field can mix electronic states, leading to changes in the absorption spectrum. For the experiments reported in this paper, the samples are embedded in a rigid matrix, so (i) is unimportant. Furthermore, because the change in dipole moment for the Q_y transition of the special pair is rather large, vida infra, (ii) appears to dominate the observed spectra, and we will make the approximation that the other effects are of secondary importance in our initial analysis.

The RCs are randomly oriented with respect to the applied electric field direction ($\mathbf{F}_{ext}$) in our experiments; consequently, the absorption spectrum is broadened when the field is applied. For a nonoriented sample in a rigid matrix, the change in absorbance due to the difference between the ground- and excited-state dipole moment as a function of transition frequency is given by (Mathies & Stryer, 1976)

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[1] Abbreviations: P870 and P960, the dimeric electron donors in *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas viridis* reaction centers, respectively; PVA, poly(vinyl alcohol); RC, reaction center.

[2] Coordinates for the *R. viridis* structure were provided by Dr. Deisenhofer and are partially refined (dated Aug 29, 1984).

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$$\Delta A(\nu) = \frac{C_\chi}{30h^2}\mathbf{F}_{int}{}^2\nu\frac{d^2(A/\nu)}{d\nu^2} \quad (1)$$

where $C_\chi = 5\Delta\mu^2 + (3\cos^2\chi - 1)[3(\mathbf{p}\cdot\Delta\mu)^2 - \Delta\mu^2]$, χ is the angle between the applied electric field direction and the polarization vector of the probing beam (see inset to Figure 1), $\mathbf{p}$ is a unit vector in the direction of the transition dipole moment being probed at frequency ν, and $\Delta\mu$ is the difference dipole moment between the ground and excited states. $\mathbf{F}_{int}$ is the actual field felt by the molecules under investigation, which is different from the applied electric field because of the dielectric properties of the environment. The magnitude and origin of this local field correction, $\mathbf{F}_{int} = f\mathbf{F}_{ext}$, have been discussed extensively (Chen et al., 1975), and it can have a substantial effect on the correct quantitative analysis of $|\Delta\mu|$. In order to clearly separate the experimental uncertainties from assumptions used to treat the local field, we express the value of $|\Delta\mu|$ as the product of f^{-1} and the observed value of $|\Delta\mu|$ assuming $\mathbf{F}_{int} = \mathbf{F}_{ext}$.

$\Delta\mu$ is a vector quantity, and it is also possible to measure the angle, ζ, between $\Delta\mu$ and the direction defined by the transition dipole moment ($\mathbf{p}$) for the associated electronic transition because of the $\mathbf{p}\cdot\Delta\mu$ term in eq 1. Note that the uncertainties in $|\Delta\mu|$ due to the local field correction have no impact on the determination of ζ. To the extent that the direction of the transition moment is known with respect to the molecular axes of the molecule(s) under consideration, a physical model for the charge separation pathway can be developed. Stark spectroscopy has been successfully applied to a wide range of organic molecules (Hochstrasser, 1973), including studies of the visual pigments by Mathies and Stryer (1976), whose experimental methods and methods of analysis we have closely followed.

Materials and Methods

Samples. *R. sphaeroides* RCs were prepared by standard methods (Schenck et al., 1982) and were embedded in poly(vinyl alcohol) films (PVA; Aldrich; average M_r 125 000). Films were typically 0.1–0.15 mm thick; the thickness of the sample in the region studied was measured with a precision caliper (accuracy ±0.005 mm). The samples were coated on both sides with semitransparent Ni electrodes (thickness typically 80 Å), each giving about 45% transmission in the visible and near-infrared regions. The sample concentration in the film was typically 1.5×10^{-4} M.

Apparatus. The samples were probed with light from a 250-W tungsten–halogen lamp passed through a $^1/_4$-m double monochrometer (resolution 10 nm). The high-voltage leads were connected to the Ni electrodes by springs. The sinusoidal ac applied field was generated by a voltage source of local design (0–5 kV). The field applied to the sample was measured with a high-voltage probe attached to the leads while the experiment was performed. The signal was detected with an RCA C30956E Si avalanche photodiode having excellent sensitivity between 500 and 1000 nm with low noise. The small ac component (ΔI) was lock-in detected at the second harmonic of the field modulation frequency (typically 1050 Hz) and was digitized along with the large dc component corresponding to the transmitted intensity (I); $\Delta I/I$ was obtained in a computer. Transmission spectra were converted to absorption spectra by recording the transmission of a suitable blank, and first and second derivatives were obtained in the computer. For polarization measurements the probing light was passed through a Glan-Thompson polarizer to produce horizontally polarized light. The angle between the electric vector of the light and the applied electric field, χ, was varied by rotating the sample about a vertical axis. The sample geometry and relevant angles are shown in the inset to Figure 1. All experiments were performed at ambient temperature (20 °C).

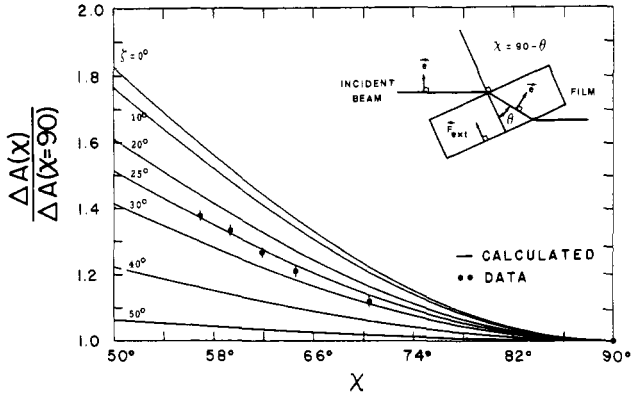

FIGURE 1: Plots of the calculated dependence of $\Delta A(\chi)/\Delta A(\chi = 90°)$ on the experimentally variable angle χ as a function of the angle, ζ, between the direction of a transition dipole moment and the difference dipole moment, $\Delta\mu$ (solid curve; eq 1). Experimental data obtained for the special pair Q_y transition (855 nm, 11 696 cm^{-1}) are indicated by the solid circles and error bars (±SD). The inset illustrates the geometry of the sample and probe beam used for Stark measurements.

Methods of Analysis. In order to quantitatively evaluate the Stark spectra, $\Delta I/I$ must be corrected for multiple passes of the light through the sample due to internal reflections by the electrodes. The reflectivity of Ni in the wavelength range of interest as a function of wavelength and χ was measured and combined with the value of the transmission of the sample to produce a small correction point by point in the computer. It is instructive to plot the dependence of ΔA on χ (eq 1), calculated for various possible angles ζ between the transition dipole moment and $\Delta\mu$, and this is shown in Figure 1. This plot demonstrates the very great sensitivity of the measurement to the value of ζ. Details of the evaluation of $|\Delta\mu|$ from the data are deferred to the next section.

Results

The Stark spectrum ($\chi = 90°$) of *R. sphaeroides* RCs in the near-infrared and visible regions is shown in Figure 2. It is qualitatively clear that the electric field has a much greater effect on the special pair Q_y absorption band than on the absorption features at 760 and 802 nm.[3] This difference has been previously noted by DeLeeuv et al. (1982). The effect is striking when compared with the second derivative of the absorption spectrum, which is also shown for comparison (Figure 2C). The second derivative is expected to resemble the Stark spectrum except for the absence of weighting by the factor $|\Delta\mu|^2$ (eq 1);[3] the line shape of the Stark spectrum in

[3] The absorption features in the 500–1000-nm range in a PVA film at room temperature are assigned to the special pair (lower energy exciton component of the Q_y band at 855 nm), the two monomer bacteriochlorophylls (overlapping Q_y and Q_x transitions at 802 and 600 nm, respectively), and two monomer bacteriopheophytins (overlapping Q_y and Q_x transitions at 760 and 540 nm, respectively). These assignments are useful but crude due to strong intermolecular interactions. Where two transitions are unresolved, we will assume that the Stark effect for each underlying transition is identical. Low-temperature experiments should resolve this uncertainty. It is possible that $\mathbf{F}_{int}$ is different at the sites occupied by each chromophore. Since it is very unlikely that the local field correction could differ by more than about 50%, the variations in the Stark effect for the different absorption bands are almost certainly dominated by differences in $\Delta\mu$. We note, however, that local variations in the dielectric properties of the protein matrix may play a fundamental role in directional charge separation.

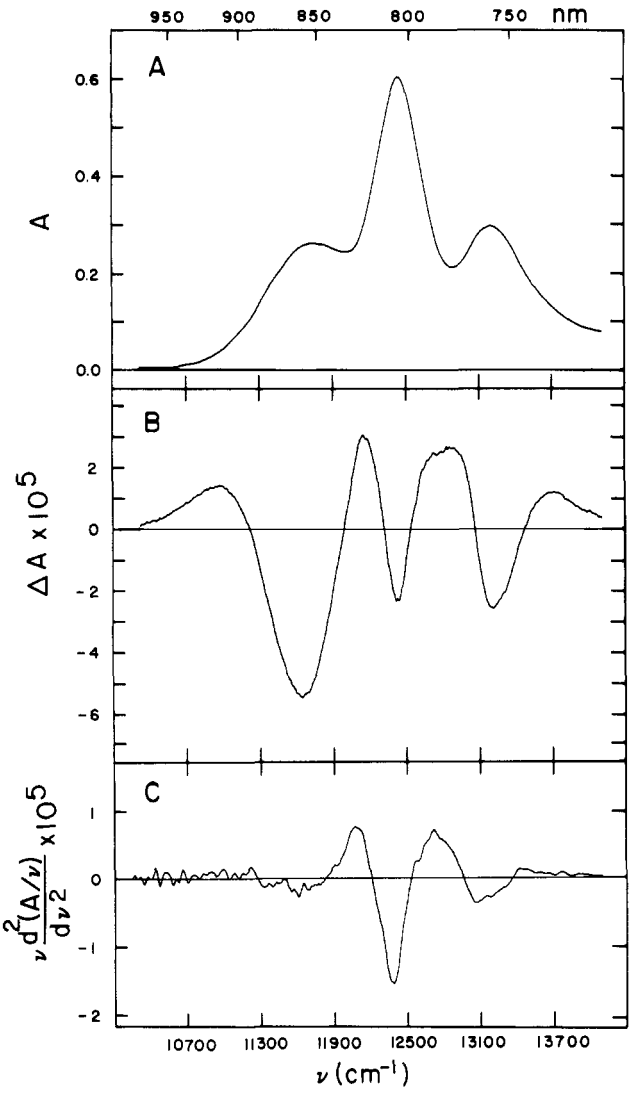

FIGURE 2: Absorption (A), Stark (B), and second derivative (C) spectra for the Q_y region of *R. sphaeroides* RCs ($\mathbf{F}_{ext}$ = 9.12 × 10^4 V/cm). The Stark data have been corrected for multiple passes of the probing light through the sample (χ = 90°).

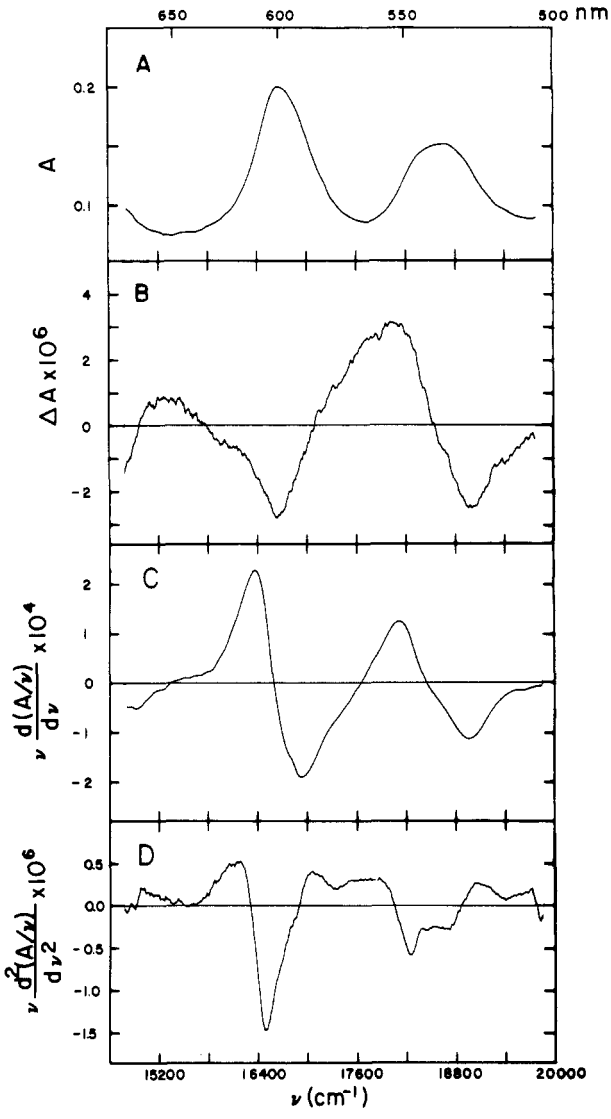

FIGURE 3: Absorption (A), Stark (B), first derivative (C), and second derivative (D) spectra for the Q_x region of *R. sphaeroides* RCs. The absorption and Stark spectra were for the same sample and conditions as in Figure 2; the derivative spectra were obtained on a solution sample whose optical density was 3 times greater than the film sample to obtain reasonable signal to noise.

this region does closely resemble a second derivative. After correction for differences in the line widths of each band, $|\Delta\mu|$ for the special pair Q_y band is a factor of approximately 2 and 3.4 times greater than for the monomeric bacteriopheophytin (760 nm) and bacteriochlorophyll (802 nm) Q_y bands, respectively.[3]

The Stark effect in the 500–650-nm (Q_x) region is shown in Figure 3. The effect is much weaker than in the Q_y region and the line shape is more complex than a simple second derivative (Figure 3D), especially for the overlapping bacteriopheophytin Q_x bands (the first and second derivative spectra in the Q_x region, parts C and D of Figure 3, were obtained on a solution sample whose optical density was 3 times that of the film sample used to obtain the Stark spectrum in order to achieve reasonable signal to noise). This may be a result of a breakdown in some of the approximations made at the outset, notably that the effect due to the dipole moment difference is large relative to that for the polarizability difference. If the polarizability difference dominates, the Stark spectrum has the line shape of the first derivative of the absorption spectrum, which is plotted in Figure 3C for comparison.

A plot of the dependence of $\Delta A(\chi)/\Delta A(\chi = 90°)$ on χ for the Q_y transition of the special pair is shown in Figure 1. This plot demonstrates that the angle between the special pair Q_y transition dipole moment and $\Delta\mu$ is 24 ± 2° (ζ for the Q_y transitions of the monomer bacteriochlorophyll and bacteriopheophytin are both approximately 0°, though the presence of overlapping bands and the relative magnitudes of the effect make this measurement less precise than at 855 nm). For χ = 90° and $\mathbf{p}\cdot\Delta\mu$ = 24°, $C_\chi = 3.50\Delta\mu^2$. $\nu[d^2(A/\nu)/d\nu^2]$ was determined most accurately by modeling the special pair Q_y absorption band as a Gaussian with full width at half-maximum = 920 cm^{-1}.[4] At the peak of the band $\nu[d^2(A/\nu)/d\nu^2]$ = 2.0 × $10^{-6}/(cm^{-1})^2$. $\Delta A(\nu)$ is determined from the experimental observables by using $\Delta A(\nu) = [-2\sqrt{2}(\Delta I/I)m]/2.303$, where ΔI is the signal recorded with the lock-in amplifier, I is the dc signal, and m is a factor correcting for multiple passes of the probing light due to the reflectance of the Ni electrodes.

[4] This approach takes advantage of the excellent signal to noise of the absorption spectrum as compared with the extremely small and thus noisy second derivative for a broad band. This approximation is flawed because the 855-nm band is not a simple Gaussian and there is substantial overlap with the 802-nm band. A more accurate line-shape analysis will be presented with low-temperature data in a subsequent paper.

Table I: Comparison of Effects of Local Field Correction for Different Models and Values of the Dielectric Constant on the Value of $|\Delta\mu|$ for P870

ϵ	approximation	f	f^{-1}	$\lvert\Delta\mu\rvert$ (D)
2	spherical cavity	1.20	0.83	8.6
4		1.33	0.75	7.7
2	Lorentz local field	1.33	0.75	7.7
4		2.0	0.5	5.2

At 855 nm (11 696 cm^{-1}) and an applied field of 9.12×10^4 V/cm, $\Delta I/I = 4.8 \times 10^{-5}$. For $\chi = 90°$, $m = [1 - (R_eT_s)^2]/[1 + (R_eT_s)^2] = 0.976$, where R_e is the reflectance of the Ni electrodes and T_s is the transmission of the sample. With the above values, $|\Delta\mu|$ is calculated to be $f^{-1}(10.3 \pm 0.7)$ D.

We have obtained preliminary Stark data for bacteriochlorophyll *a* and bacteriopheophytin *a* monomers in polystyrene ($\epsilon = 2.6$) (Lockhart and Boxer, unpublished data). For *both* molecules $|\Delta\mu|$ for the Q_y transition was found to be approximately $f^{-1}(2–3)$ D and ζ was approximately 0°. The Stark spectrum of the Q_x region of bacteriopheophytin *a* approximates the shape of the first derivative of the absorption band.

Discussion

The value $|\Delta\mu| = f^{-1}(10.3 \pm 0.7)$ D for the Q_y transition of the special pair is considerably larger than for the monomer bacteriochlorophylls and bacteriopheophytins in the RC[3] or for the pure monomers in polystyrene. Both $|\Delta\mu|$ and ζ are comparable for the monomeric bacteriochlorophyll in the RC and in polystyrene. The magnitude of $|\Delta\mu|$ for the monomer bacteriopheophytin in the RC is about twice that in polystyrene; however, ζ is comparable. Furthermore, the line shape of the Stark spectrum for the Q_x transition in the RC is a property of pure bacteriopheophytin *a*. The most striking effects are the differences in both $|\Delta\mu|$ and ζ for the Q_y transition of the special pair compared to monomer bacteriochlorophyll *a* in the RC or pure in polystyrene. Ignoring possible differences in f (see below), $|\Delta\mu|$ for the special pair is almost $f^{-1}(7)$ D larger than for monomer bacteriochlorophyll *a*, a very large difference, and $\Delta\mu$ is rotated relative to the Q_y axis in the special pair as compared with monomer bacteriochlorophyll *a*. In the following discussion we propose a physical model to explain these differences.

The value of f can greatly affect the correct result for $|\Delta\mu|$. The chromophores occupy cavities in irregularly shaped objects (the reaction center protein complexes) whose dielectric properties are not homogeneous and are not known exactly, and these are embedded in PVA whose dielectric constant is 2.25.[5] Since the structure for the RC protein is emerging in great detail (Deisenhofer et al., 1985; Michel et al., 1986), it should be possible to estimate the value of f from the structure [see, e.g., Honig et al. (1986)]; however, for the present discussion we outline the likely range of values for f in order to get a feeling for the actual value of $|\Delta\mu|$. Two widely used formulations for the local field correction are the spherical cavity approximation [$f = 3\epsilon/(2\epsilon + 1)$] and the Lorentz local field correction [$f = (2 + \epsilon)/3$], where ϵ is the bulk dielectric constant that we take to include both the protein and PVA. Values of f using these approximations and dielectric constants of 2 and 4 are given in Table I, illustrating the resulting range of values for $|\Delta\mu|$. A treatment related to the spherical cavity approximation was used to model f for retinal in bacteriorhodopsin embedded in PVA (an ellipsoidal cavity was more appropriate for that chromophore). This gave reasonable values for $\epsilon = 2$ (Ponder, 1983), so we will use the value $|\Delta\mu| = 8.6$ D (Table I) for the purpose of discussion.

There is no direct information on the ground-state dipole moment of the special pair or for bacteriochlorophyll. Since $\Delta\mu$ is a vector quantity, any interpretation of our data depends on both the magnitude and direction of the permanent dipole moments in the ground and excited states. If the ground-state dipole moment for the special pair were very small compared to 8.6 D, then $|\Delta\mu|$ is dominated by the dipole moment of the excited state.[6] To obtain a physical feeling for such a result, a full charge separated between the monomers comprising the special pair would produce a dipole moment of about 34 D (the Mg–Mg distance in the *R. viridis* special pair structure[2] is 7.1 Å, and we crudely approximate the dipole moment by assuming point charges at the center of each macrocycle). If the ground state is dipolar and its dipole moment direction is substantially different from that in the lowest excited singlet state, the magnitude of $\Delta\mu$ is less straightforward to interpret.

The observation that the transition dipole moment for excitation of the lowest singlet state of the special pair and $\Delta\mu$ are not parallel for the special pair offers a basis for proposing a physical model in which charge is separated between the macrocycles comprising the special pair (the planes of the monomers are nearly parallel, separated by about 3.5 Å, and overlap at ring I). A full experimental determination of the direction of the special pair Q_y transition dipole moment relative to the special pair molecular axes has not been published to date. From the limited analysis of single crystal absorption data that are available (Zinth et al., 1985), it appears that the special pair Q_y transition at 960 nm in *R. viridis* crystals is polarized approximately along the direction of the vector sum of the *Y* axes of the monomers comprising the special pair, as expected (the *Y* axis is defined by the line connecting the nitrogen atoms in rings I and III [see, e.g., Moog et al. (1984)]. Taking this direction for the Q_y transition of the special pair in *R. sphaeroides*, we can calculate the angle ζ between the Q_y transition moment and a vector connecting the central Mg atoms of the monomers comprising the special pair. This angle is approximately 25°, which agrees well with the angle we have measured experimentally (Figure 1). By contrast, a vector connecting a point halfway between the Mg atoms of the special pair and the central Mg atom of the monomeric bacteriochlorophyll on the L side of the RC makes an approximate angle of 45° with the assumed Q_y direction. In order to go beyond this level of analysis, detailed calculations are required, and we trust that the data presented in this paper will stimulate such analyses.

On the basis of our very simple analysis, we suggest that charge is separated initially between the macrocycles comprising the dimer, rather than between the special pair and the monomer bacteriochlorophyll. Of course, the Stark data for a nonoriented sample provide only information on the angle ζ that defines a cone (the transition dipole moment defines a line). Nonetheless, the data reported in this paper lead us to the hypothesis that the first charge-separated state in bacterial photosynthesis is formed directly upon photoexcita-

[5] Due to the presence of polarizable groups in the protein, dipoles will be induced by the externally applied electric field, and the induced dipoles could affect the local dielectric properties near the chromophores. However, the fields used in these experiments are relatively small so that this effect is probably negligible. This is supported by the observation that $|\Delta\mu|$ and ζ for pure bacteriochlorophyll *a* in polystyrene and for the monomeric bacteriochlorophyll in the RC are similar.

[6] Even if the ground-state dipole moment of monomeric bacteriochlorophyll were substantial, the dipole moment for the dimeric special pair could be quite small.

tion. Stark spectra at low temperature, for oriented samples, for *R. viridis* RCs, and for chlorophylls in simpler protein environments, will be reported shortly.

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