Electrooptical Properties of Different Redox States of Native and Modified Reaction Centers of *Rhodobacter sphaeroides*

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The Stark spectra of selectively and nonselectively BPheo → Pheo exchanged reaction centers are presented and compared to those of native reaction centers. It is shown that Pheo, when incorporated into the reaction center protein, shows an almost pure second-derivative shaped Stark spectrum indicating that absorbance changes are mainly caused by the interaction between the electric field and the change in permanent electric dipole moment $|\Delta\mu|$ upon excitation. $|\Delta\mu|$ is estimated to be about 1.5 D/*f*, which is substantially smaller than that of the native BPheo pigment. By comparing the spectra of open reaction centers to those of reaction centers in the $P^+\Phi_AQ_A^-$ state, evidence for a difference in polarizability between the ground and the excited state of the BPheo is found. Although the difference polarizability is not large enough to appreciably influence the Stark spectrum of randomly oriented molecules induced by an external electric field, it is argued that it *is* large enough to necessitate its inclusion in the analysis of electrochromic bandshifts induced by an internal electric field. The near-equal optical energies and electrooptical parameters of the Pheos reconstituted in the active and inactive branch, as well as their electrochromic bandshift upon $P^+\Phi_AQ_A^-$ formation (which is much smaller than for the native BPheo pigments), are discussed in terms of the interaction of the Pheos with internal electric fields from their environment.

1. Introduction

In the past, much attention has been devoted to Stark spectroscopy of the primary donor band in photosynthetic reaction centers from purple bacteria (see, for example, refs 1−3). It was shown by measuring the Stark effect spectrum at 1.5 K in frozen glycerol/buffer samples[2] that the Stark line shape of the primary donor band is not accurately described by a pure second-derivative of the absorption line shape, indicating that, unlike the monomeric bacteriochlorophylls (BChls) and bacteriopheophytins (BPheos) incorporated in the reaction center, the primary donor possesses quite a large polarizability term, which gives rise to a first-derivative of absorption contribution in the Stark spectrum. Additionally, the Stark effect indicates that the primary donor has a sizable difference dipole moment, about 2−3 times higher than that of the monomeric pigments. This large difference dipole has been attributed to the admixture of charge-transfer states, but this explanation predicts an electric field dependence of the Stark line shape that is not observed experimentally.[2,5] Prompted by the large polarizability of the dimer it was suggested that the large difference dipole is, in fact, an induced dipole due to the protein matrix field felt by the BChl dimer[2].

The Stark line shape of the accessory BChl and BPheo bands has drawn much less interest than that of the primary donor. The main reasons for this lack of attention probably are that the lineshapes are almost pure second derivatives and the difference dipole moments about equal to those in monomeric pigments in solution,[6] while the C_2-symmetry in the reaction center gives rise to two spectroscopically nearly identical pigments. For instance, it is difficult to compare the difference dipole moment associated with the active and the inactive BPheo band. Steffen et al.[7] recently showed that the electric properties of the surrounding protein matrix are quite different at the location of the active and the inactive pigments, by calculating the field at each pigment caused by the radical pair $P^+\Phi_AQ_A^-$ (P, primary donor; $\Phi_{A,B}$, bacteriopheophytin in the active and inactive cofactor chain, respectively; Q_A, quinone electron acceptor) and using this field to calculate the expected bandshift when the pigments are placed in a vacuum. The ratio between the calculated band-shift and the experimentally observed one is a measure for the dielectric screening by the surrounding protein matrix, provided the pigment under study has a negligible polarizability and the dielectric constant is isotropic.

To learn more about the dielectric properties of the protein matrix and the electrooptical properties of the monomeric pigments, we compare in this work the Stark and absorption spectra of three types of reaction centers, native ones with BPheo in both the active and the inactive electron transport chain, $\Phi_{A,B}$-exchanged reaction centers where both BPheos are removed chemically and replaced by plant pheophytins (Pheos),[8,9] and Φ_B-exchanged reaction centers where the inactive BPheo pigment is selectively replaced by a Pheo, leaving the native BPheo in the active branch unchanged.[9] Especially the Φ_B-

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exchanged reaction centers offer a chance to examine the isolated absorption band and Stark effect of $BPheo_A$, because the absorption of the exchanged inactive pigment is blue-shifted by about 90 nm with respect to the native BPheo,[9] completely removing the spectral overlap between the two pigments.

Another way to study the optical properties of the monomeric pigment bands is to compare the spectra of neutral, open, reaction centers to those of the reaction centers in the charge-separated state $P^+\Phi_AQ_A^-$. The well-known bandshifts that occur[10] are primarily caused by two effects: the change in dipole-dipole (exciton) interaction between the pigment and the primary donor when an electron is removed from the latter, and the electric (Coulomb) field caused by the charge-separated state $P^+\Phi_AQ_A^-$, which shifts the molecular energy levels of the pigments due to its interaction with the difference electric dipole moment. It is believed that for both the BChl and the BPheo the electrostatic interaction with the Coulomb field of the radical pair is the main cause of the bandshifts. Especially for the BPheo, the dipole−dipole interaction with the primary donor is very weak as can be expected from the large distance between the two pigments.[11] Indeed, in the triplet-minus-singlet spectra of the reaction centers, which are very sensitive to dipole−dipole interactions since both the triplet and singlet state are uncharged, no significant coupling between P and BPheo is observed.[12,13]

Steffen et al.[7] showed that the experimental shifts of the BPheo absorption bands caused by the radical-pair electric field are much smaller than those expected from the electric field, calculated in a vacuum from the charge distribution of the radical ions as measured by ENDOR spectroscopy and the difference in permanent dipole moment of the ground and excited state of the BPheos as measured by Stark spectroscopy. Clearly the lower experimental shift is caused by dielectric screening of the protein surrounding the BPheo pigment, lowering the effective amplitude of the electric field. Analyzing the bandshifts for both BPheos and comparing them to the bandshifts expected in a vacuum, Steffen et al. concluded that the dielectric constant for the active branch of the reaction center is about 3 times higher than for the inactive branch.

2. Materials and Methods

The setup used for measuring the Stark spectra has been described in ref 14, the only difference being that the experiments described here were performed with the sample immersed in liquid helium pumped below the λ-point to 1.5 K to ensure an optical path undisturbed by helium bubbles. At this low temperature the bands are narrower and more structure can be resolved than at 77 K. The setup is comparable to other, conventional, low-temperature Stark spectrometers except for the placement of the monochromator; we place the monochromator after the sample while in most other cases it is placed before the sample. This configuration allows us to measure the Stark spectrum and create a substantial amount of radical pairs at the same time. For controlling the light intensity, a diaphragm is placed between the high-intensity light source and the sample.

The spectra of open (state $P\Phi_AQ_A$) and charge-separated reaction centers (state $P^+\Phi_AQ_A^-$) were obtained by changing the intensity of the broad-band excitation/detection light beam. We could vary the total broad-band intensity between 4.5 and 450 mW/cm^2. At the lowest intensity we observed no bleaching of the primary donor band, indicating that in this case we measure the spectra of the open reaction centers. Using the full light intensity resulted in 85−100% of full bleaching of the primary donor band depending on the optical density of the sample. For those samples where full bleaching was not obtained, due to the competition between the photoinduced creation of radical pairs and their recombination with a time constant of about 25 ms,[15] we subtracted the spectra of the open reaction centers (low light intensity) from the spectra originating partially from the charge-separated reaction centers and partially from the nonbleached, open, reaction centers (high light intensity), until no observable absorption and Stark effect was left in the primary donor region.

The reaction centers from the purple bacterium *Rhodobacter (Rb.) sphaeroides* were isolated as described.[16] The method used for the BPheo → Pheo exchange is a modification of the method developed by Meyer and Scheer[8] and is described in ref 9. The samples were diluted with 50−60% (w/v) glycerol to ensure a clear glass upon cooling. The final optical density was 0.1−0.7 in the 83 μm path length cuvette.

The spectra were fitted using the method described in ref 14. We found that the choice of the line shape used for fitting considerably influences the resulting Stark parameters (see also Discussion). The fit results are only reliable when for each pigment the line shape used for fitting indeed represents its experimental optical line shape. Theoretically, these line shapes including vibronic transitions can be satisfactorily described,[17,18] but the resulting expressions are far too complicated to use in an automatic fitting procedure. When the absorption of each pigment is approximated by one single Gaussian (3 fit parameters), the fits are unsatisfactory so one has to choose a more complicated line shape. Fits using two Gaussians (6 parameters) for each pigment give much better results but suffer from too many dimensions in the parameter space so that, without additional constraints, the fits are unstable. In our experience using a single skewed Gaussian band[19] (4 parameters) for each pigment is an acceptable compromise: the parameter space is more suitably restricted and the line shape mimics the vibrational progressions quite well. The skewed Gaussian absorption line shape[19] is given by

$$\frac{\epsilon(\nu)}{\nu} = \exp\left(-\ln(2)\left[\frac{\ln(1 + 2b(\nu - \nu_{max})/\Delta\nu)}{b}\right]^2\right) \quad (1)$$

where $\epsilon(\nu)$ is the extinction at energy ν, ν_{max} the energy of the maximum of the absorption band (in wavenumbers), $\Delta\nu$ the width of the absorption band, and b the skewing parameter. If $b = 0$, eq 1 reduces to a simple Gaussian, increasing b leads to a more-and-more asymmetric band shape for which the sign of b determines whether the broader wing is at the high- or low-energy side of the band.

For plotting and comparing spectra and parameters all measured absorption spectra were scaled according to the total absorption in the BChl region of the spectra to be compared, obtained by integrating the fitted bands in the 800 nm region. For fitting the spectra, one band was used to describe the primary donor region, and one band for each monomeric pigment. Only the isolated $Pheo_B$ band in the Φ_B-exchanged reaction centers could not be adequately described by a single band so two bands were used instead. One very broad skewed Gaussian was used to account for base line effects. This band was not allowed to contribute to the Stark spectra.

3. Results

In Figure 1 the absorption, absorption second-derivative, and Stark spectra are shown of reaction centers of *Rb. sphaeroides* R-26 in the open and in the charge-separated state $P^+\Phi_AQ_A^-$.

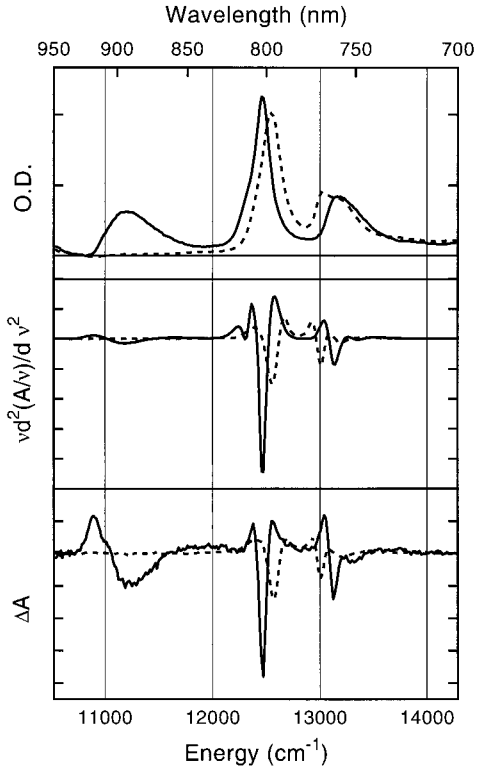

Figure 1. Absorption, absorption second-derivative, and Stark spectra of reaction centers of *Rb. sphaeroides* R-26 at 1.5 K. The spectra are recorded for open reaction centers (solid line) and for reaction centers in the charge-separated $P^+\Phi_AQ_A^-$ state (dashed line). The latter state was created by increasing the intensity of the measuring light until the light was (almost) saturating. The externally applied electric field was modulated at 700 Hz and had an RMS value of 6.17×10^6 V/m. The angle χ was 90°.

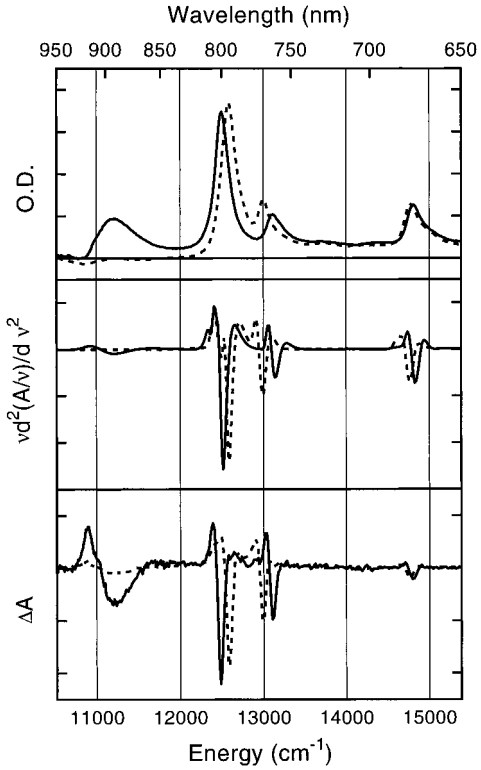

Figure 2. Absorption, absorption second-derivative, and Stark spectra at 1.5 K of reaction centers of *Rb. sphaeroides* R-26 for which the bacteriopheophytin in the inactive branch was replaced by plant pheophytin. The spectra are recorded for open reaction centers (solid line) and for reaction centers in the charge-separated $P^+\Phi_AQ_A^-$ state (dashed line). To record the latter spectrum, the intensity of the light beam was increased until the light was (almost) saturating. The externally applied electric field was modulated at 700 Hz and had an RMS value of 6.17×10^6 V/m. The angle χ was 90°.

The spectra of the open reaction centers are similar to spectra measured at 1.5 K in ref 2. It is seen that, in the 800 nm region, where the accessory BChls absorb, and in the 760 nm region, where the BPheos absorb, the shape of the Stark spectrum coincides very well with the shape of the second-derivative of the absorption spectrum. As noted first by Middendorf *et al.*,[2] the primary donor region around 890 nm cannot be accurately described by the second-derivative absorption line shape. This discrepancy has been explained by assuming that the primary donor, in addition to a large difference dipole moment, also has a large difference polarizability. For reaction centers in the charge-separated state, one sees the bleaching of the primary donor absorption band around 890 nm and the commonly observed bandshifts in the BPheo and accessory BChl regions. At first glance the induced changes agree with the changes observed in the Stark spectrum.

Figure 2 shows the same series of spectra for Φ_B-exchanged reaction centers. The absorption spectra in Figures 1 and 2 differ mainly in the appearance of the $Q_y(0-0)$ absorption band around 670 nm of the $Pheo_B$ molecule that is selectively introduced in the inactive electron transport chain of the reaction centers. Furthermore the "native" BPheo band at 760 nm shows changes because of the disappearance of the $BPheo_B$ absorption, leaving only the absorption band of the active $BPheo_A$. The Stark spectrum shows an additional signal in the Pheo absorption region.

In Figure 3 again the absorption, second-derivative absorption, and Stark spectra are shown, this time for the open and charge-separated $\Phi_{A,B}$ reaction centers where both BPheos are exchanged for plant Pheo molecules.

The differences between the absorption spectra we observe in Figures 1−3 are similar to those reported by Franken *et al.*[9] who measured the same spectra at 10 K.

All data were fitted using a nonlinear least-squares algorithm as in ref 14. The absorption spectrum was fitted to a sum of skewed Gaussians, and the Stark spectrum was simultaneously fitted to a sum of the zeroth- first- and second-order derivatives of the bands. Because, by visual inspection, the Stark line shapes are dominated by the second derivative of the absorption spectrum, initially only the second-order term was used. When this did not produce an acceptable fit the first-order and zeroth-order terms were added. All fits were performed letting the parameters completely free to evolve in any direction. To calculate $|\Delta\mu|$ from the fit results, one needs to know the angle ζ between $\Delta\mu$ and the transition dipole moment:

$$C\chi = |\Delta\mu|^2[5 + (3\cos^2\chi - 1)(3\cos^2\zeta - 1)] \qquad (2)$$

where χ is the experimental angle between the polarization of the measuring light and the applied magnetic field, and C_χ is the amplitude of the second-derivative contribution to the Stark spectrum. Because measuring the angle ζ is difficult in glycerol/buffer samples due to scattering,[3] we used the values found in PVA[6] instead: 20° for the BChl band, 10° for the BPheo band, and 45° for the primary donor (the last value taken from the more elaborate analysis in ref 2). We compared the relation between the Stark intensity of the Pheo and BPheo bands and the angle between the polarization direction of the measuring light and the applied electric field. We found that for Pheo the angle ζ is at least as small as for BPheo, so essentially close to

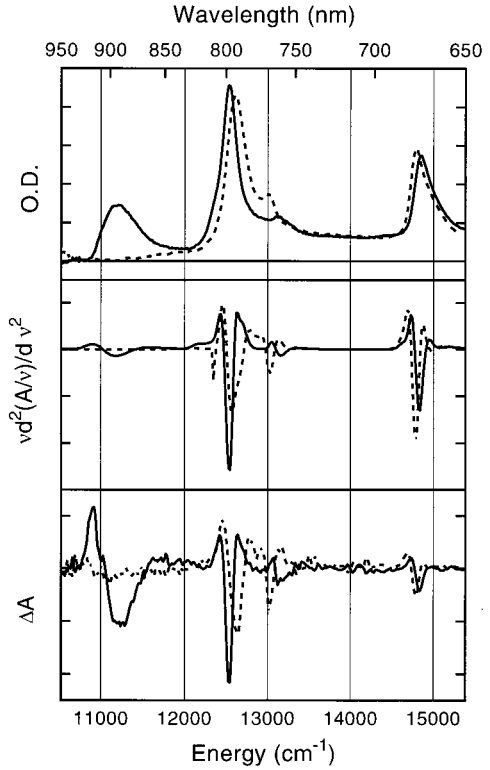

Figure 3. Absorption, absorption second-derivative, and Stark spectra at 1.5 K of reaction centers of *Rb. sphaeroides* R-26 for which both bacteriopheophytins were replaced by plant pheophytin. The spectra are recorded in open reaction centers (solid line) and in reaction centers in the charge-separated $P^+\Phi_AQ_A^-$ state (dashed line). Further conditions as in Figure 2.

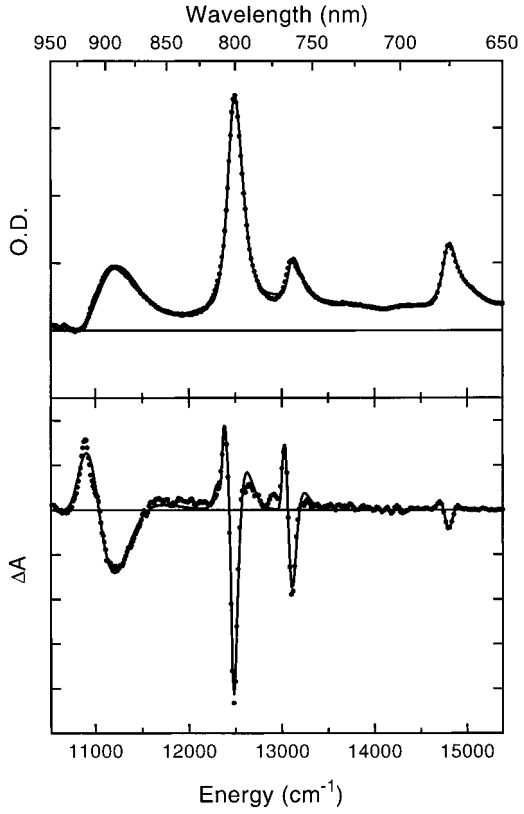

Figure 4. Typical result of the least-squares fit procedure. Represented are the absorption spectrum and Stark effect data (dots) and the fitted spectra (solid line) for Φ_B-exchanged reaction centers of *Rb. sphaeroides*. The experimental spectra are the same as those in Figure 2; the fit parameters are given in Table 1.

zero. Because varying the angle ζ between 0° and 10° leads to changes in the value for $|\Delta\mu|$ of only about 1.5% (much less than the experimental uncertainty in $|\Delta\mu|$) we used $\zeta = 0$ for the Pheo bands.

The results from all fits are given in Table 1. An example of a fit is shown in Figure 4, all other fits matched the data in a comparable way.

4. Discussion

4.1. Φ_B- and $\Phi_{A,B}$-Exchanged Reaction Centers. *4.1.1. Line Shape and Electrooptical Parameters.* The second-derivative absorption spectrum of Φ_B-exchanged reaction centers shows similar line shapes and amplitudes for the Pheo and BPheo bands (Figure 2). The corresponding Stark bands, however, show a large difference in amplitude, indicating that the electrooptical parameters of the two bands are quite different. Both the line shapes of the Bpheo and the Pheo Stark spectra are dominated by the second-derivative absorption line shape (Figure 5), indicating that the Stark effect is mainly caused by the difference in permanent dipole moment between the ground and excited state $|\Delta\mu|$. From comparing the intensities of the Stark spectrum and the absorption second derivative in Figure 2, $|\Delta\mu|$ is clearly much larger in Φ_A than in $Pheo_B$.

Stark effect measurements on isolated BPheo and BChl in solution showed that the Stark effect is accurately described by the second-derivative absorption line shape with a change in dipole moment $|\Delta\mu|$ of 2−3 D.[3,4] Krawczyk[20] reported that for Chl in solution the $Q_y(0-0)$ (670 nm) band is accurately described by a pure second-derivative shaped Stark spectrum, with a $|\Delta\mu|$ of 1 D/*f*,[21] a factor of 2−3 lower than that for BChl.

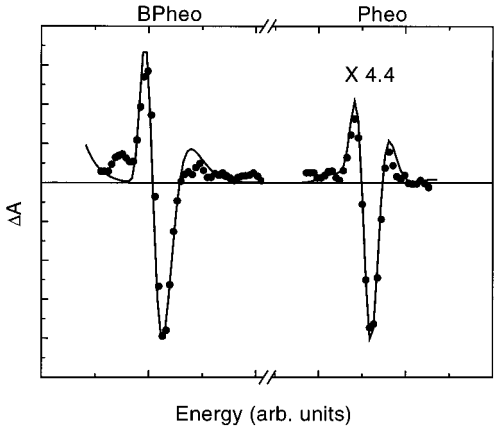

Figure 5. Stark (dots) and absorption second-derivative (solid line) spectra of the isolated regions of the BPheo in the active electron transport chain (left) and the Pheo in the inactive chain (right), as measured in the Φ_B-exchanged reaction centers of *Rb. sphaeroides* R-26. For ease of comparison, the (much smaller) Pheo Stark effect is scaled such that its amplitude equals that of the BPheo band. All experimental conditions are the same as those in Figure 2. The second-derivative line shapes are scaled to the minimum of the Stark effect in both bands.

For fitting the $Q_y(0-0)$ absorption of the exchanged Pheo, we needed two skewed Gaussian bands, probably because the transition overlaps with the low-intensity transition around 680 nm found in native reaction centers. Table 1 shows that the electrooptical parameters of both bands are about equal.

4.1.2. Differences between $BPheo_{A,B}$ and $BChl_{A,B}$. The fit results show that $|\Delta\mu|$ is about 4.2 D/*f* for the native, inactive $BPheo_B$ (absorbing around 750 nm) and 3.5 D/*f* for the active

TABLE 1: Results from All the Least-Squares Fits Described in the Text[a]

ν (cm^{-1} (nm))	A[b]	σ (cm^{-1})	b	$A\chi$ $(m/V)^2$	Tr($\Delta\alpha$) ($Å^3/f^2$)	$\lvert\Delta\mu\rvert$ (D/*f*)
			Native RCs (Open)			
13319 (751)	44	250	0.49			4.2
13153 (760)	44	161	0.07			3.5
12461 (803)	149	145	0.21			2.2
12342 (810)	51	132	−0.41			2.1
11231 (890)	208	507	0.30	-3×10^{-7}	590	5.5
			Native RCs (Charge Separated)			
13189 (758)	63	242	0.54			2.4
13022 (768)	37	137	0.08			2.5
12625 (792)	54	203	0.27			3.3
12525 (798)	146	190	−0.20			2.2
			Φ_B (Open)			
14875 (672)	42	406	0.22			1.8
14805 (676)	17	132	0.1			1.3
13129 (762)	27	150	0.32			3.1
12494 (800)	178	167	0.37			2.2
12380 (808)	22	95	−0.26			2.0
11235 (890)	210	512	0.31	-3×10^{-7}	425	5.8
			Φ_B (Charge Separated)			
14802 (676)	46	318	0.50			1.2
14770 (677)	13	128	0.12			1.4
13001 (769)	29	126	0.17			2.5
12590 (794)	164	157	0.35			2.1
12483 (801)	36	93	−0.17			1.8
			$\Phi_{A,B}$ (Open)			
14908 (671)	106.4	369	0.28			1.5
15836 (674)	34	140	0.15			1.4
13187 (758)	18	215	0.40			3.3
12561 (796)	114	162	0.11			2.2
12497 (800)	86	179	−0.36			2.3
11219 (891)	276	472	0.25	-6×10^{-7}	590	5.5
			$\Phi_{A,B}$ (Charge Separated)			
14866 (673)	112	336	0.26			1.7
14784 (676)	34	120	−0.01			1.3
13031 (767)	18	134	0.05			3.1
12656 (790)	118	170	0.20			2.7
12551 (797)	82	134	0.0			2.1

[a] Blanks indicate that the corresponding term was not used in the fit. [b] The total intensity of the band as determined by the integral over the complete line shape scaled such that the sum of the two accessory BChls BChl is 200 in each spectrum. The integration facilitates comparing the intensities for bands with different skewing parameters.

$BPheo_A$ (absorbing around 760 nm), while the exchanged Pheos both show a $|\Delta\mu|$ of about 1.3−1.8 D/*f*. The values reported here for $|\Delta\mu|$ in the BPheo band are somewhat larger than those reported in ref 7, where $|\Delta\mu|$ was found to be 2.7 D/*f* for $BPheo_B$ and 3.3 D/*f* for $BPheo_A$. In the latter work, $|\Delta\mu|$s in the BChl region of 2.5 and 2.9 D/*f* for $BChl_B$ and $BChl_A$, respectively, were reported while we find $|\Delta\mu| = 2.2-2.3$ D/*f* for both BChls. Thus, our results not only show a larger difference between the two BPheos but also a difference between the BPheo and the BChl bands. It is not clear whether the differences between the results in ref 7 and our work are physically significant, because the parametrization of the absorption spectrum in ref 7 is different from ours, and the sample thickness in glycerol/buffer samples is difficult to determine. Measurements at 77 K in PVA films[6] resulted in $|\Delta\mu| = 2.1$ D/*f* for the BChl band and $|\Delta\mu| = 3.5$ D/*f* for the BPheo band. Comparing our results to the 77 K results from ref 6 is, however, difficult since the local field correction factor *f* is temperature dependent, and at 77 K the absorption bands of corresponding pigments in the active and inactive branch are not resolved, so only one band is used for fitting each absorption region. Note that the large difference in $|\Delta\mu|$ between the BPheos and BChls that we find from our data *is* confirmed by the data recorded at 77 K.

4.1.3. Yield of Pigment Exchange. In the spectra of the $\Phi_{A,B}$-exchanged reaction centers almost all BPheo is exchanged as is seen from the absorption as well as the Stark spectrum. The Stark effect of the BPheo band in $\Phi_{A,B}$-exchanged reaction centers shows less than 20% of the intensity in the Φ_B-exchanged reaction centers. Thus, if we assume that in the latter about 50% of the BPheos are exchanged, the total replacement is about 90% in the $\Phi_{A,B}$-exchanged reaction centers, in agreement with the results in ref 9.

4.1.4. $Pheo_A$ and $Pheo_B$. The Pheo Q_y absorption is clearly larger for $\Phi_{A,B}$-exchanged reaction centers than for Φ_B-exchanged sample. However, scaling the Pheo absorption band and shifting it slightly leads to two almost identical bands in both cases (Figure 6). When the Stark spectra are scaled and shifted in the same way both Stark spectra match almost perfectly, indicating that the electro-optical parameters for both Pheos are nearly identical (see also Table 1). The almost identical Pheo bandshape in the Φ_B- and $\Phi_{A,B}$-exchanged samples indicates that in the Q_y region the two Pheos are spectroscopically very similar, unlike the two native BPheo that are shifted about 170 cm^{-1} relative to each other. This conclusion is in agreement with the smaller and equal value of $|\Delta\mu|$ for the two Pheos, as the difference in the absorption wavelength of the two native BPheos is thought to arise from electrostatic interactions between the difference dipole moment and the protein matrix field,[7] shifting the energy difference between the ground and excited state. The unequal Q_y energies

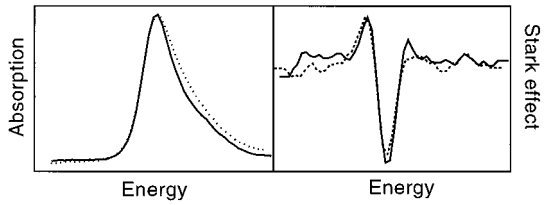

Figure 6. Plot of the absorption (left part) and Stark (right part) spectra of the Pheo Q_y-region for the Φ_B- (solid line) and $\Phi_{A,B}$-exchanged (dotted line) reaction centers of *Rb. sphaeroides* R-26. The absorption spectrum of the Φ_B-exchanged reaction center was scaled until the best overlap with the spectrum of the $\Phi_{A,B}$-exchanged reaction center was achieved. Subsequently the Stark spectrum of the Φ_B-exchanged reaction centra was scaled by the same factor. It is concluded that both Pheos are spectroscopically almost identical and possess nearly equal electrooptical parameters (see text).

of the native BPheos then arise from a different matrix field in the BPheo pocket of the inactive and active chain and from the difference in $|\Delta\mu|$ between the two pigments. Because the$|\Delta\mu|$s are much smaller and virtually equal in the case of the two Pheos, the resulting shifts in the absorption wavelength are smaller and more alike.

4.1.5. BChl Region. The differences in the accessory BChl region around 800 nm between the Stark spectra of the native, Φ_B- and $\Phi_{A,B}$-exchanged reaction centers can all be explained by the changes appearing in the optical absorption spectrum and hence in its second derivative, without changing the electrooptical parameters. The differences in the absorption around 800 nm most probably arise from a change in the dipole−dipole coupling between the BChls and the BPheo/Pheo, because of the structural and energetic differences between BPheo and Pheo.

4.2. Open versus Charge-Separated Reaction Centers. *4.2.1. Dielectric Screening.* In the work of Steffen *et al.*[7] it was demonstrated that, by comparing the calculated electrochromic shift with the experimentally observed one, an estimate can be made for the effective relative dielectric constant ϵ_{eff}. The samples used in the present work offer a possibility to check the results of Steffen *et al.* by measuring and comparing the electrochromic bandshifts in native, Φ_B- and $\Phi_{A,B}$-exchanged reaction centers.

As mentioned above Steffen *et al.* reported $|\Delta\mu| = 3.3$ D/*f* for the active BPheo and 2.7 D/*f* for the inactive BPheo, while we find 3.5 D/*f* for the active BPheo and 4.2 for the inactive one.

From the fact that the calculated electric field due to $P^+\Phi_A Q_A^-$ is about 2.7 times *larger* at the location of the active Bpheo than at the inactive pigment[22] and the fact that $|\Delta\mu|$ is also *larger* for the active than for the inactive BPheo, in combination with the observed bandshifts, which are about equal for both BPheo bands, Steffen *et al.* concluded that the effective dielectric constant is roughly 3 times larger in the active electron chain than in the inactive chain. However, because we find that $|\Delta\mu|$ is substantially *larger* for the *inactive* BPheo than for the active BPheo we expect the difference in ϵ_{eff} to be substantially smaller than that estimated in ref 7.

We calculated ϵ_{eff} for both BPheo bands in the native reaction centers using the same methodology as Steffen *et al.*[7] (see Appendix), but our own observed bandshifts and values for $|\Delta\mu|$. The results are $\epsilon_{eff} = 3.7$ for the active chain (where Steffen *et al.* find 4.5) and $\epsilon_{eff} = 2.1$ for the inactive chain (1.6 in ref 7). So, when using Steffen's method of analysis, our results *qualitatively* agree with those from ref 7 in the sense that the active chain exhibits a larger ϵ_{eff} than the inactive one.

The $Pheo_{A,B}$ band in the completely exchanged reaction centers merely shifts upon formation of $P^+\Phi_A Q_A^-$ and hardly changes shape (see the middle part of Figure 2, the second derivative of the absorption band). When shifting one spectrum to have it fall on top of the other, the two bands are nearly identical (not shown). This observation indicates that the electrochromic bandshifts for both Pheo bands are practically equal. Calculating both bandshifts by taking the electrostatic field due to $P^+\Phi_A Q_A^-$ from ref 7 and taking into account different values of ϵ_{eff} at the position of both pigments calculated above, we find a shift of 37 and 46 cm^{-1} for $Pheo_B$ and $Pheo_A$, respectively.[23] When we use the values found for ϵ_{eff} by Steffen *et al.*[7] instead, the resulting bandshifts are 47 and 61 cm^{-1}, respectively. The experimentally observed bandshift for the whole band is 50 cm^{-1}.

In the above discussion effects due to the polarizability of the pigments were neglected since $\Delta\alpha$ is generally assumed to be small for the monomeric pigments in the reaction center. We will, however, show below that upon $P^+\Phi_A Q_A^-$ formation the pigments undergo a substantial change in the difference permanent dipole moment due to the interaction between the internal electric field and the difference polarizability $\Delta\alpha$. Apparently, for BPheo *in vivo*, $\Delta\alpha$ is substantial and should be taken into account when calculating ϵ_{eff} from the bandshifts. In the Appendix we extend the treatment of Steffen *et al.*,[7] incorporating a term with (scalar) $\Delta\alpha$, and show that for reasonable estimates of $\Delta\alpha$ the effect on the bandshifts (and therefore on the resulting ϵ_{eff}) may be considerable.

4.2.2. The Difference Polarizability of (B)Pheo. Figure 1 shows that, while the BPheo band (around 760 nm) is red-shifted upon creation of the radical pair, the amplitudes of the absorption band and the second derivative of the absorption band are hardly influenced. Unexpectedly, the amplitude of the Stark effect in this region drops considerably when $P^+\Phi_A Q_A^-$ is formed. In principle, this change in Stark amplitude can be caused either by a change in the absorption lineshape or by a change in the electrooptical parameters of the BPheo. The equal amplitudes of the two absorption second-derivative bands suggest that the change in amplitude of the Stark effect is not caused by a change in the absorption line shape. To quantitate this suggestion we have fitted the $P^+\Phi_A Q_A^-$ spectrum by using the values for $|\Delta\mu|$ as derived from the fit of the open reaction centers and allowing only the parameters that describe the optical line shape to change. Although the absorption spectrum could be nicely fitted in this way, the method did not result in a satisfactory fit of the Stark spectrum. To achieve a good fit of the Stark spectrum we had to allow a change of $|\Delta\mu|$ as well. Table 1 shows that, for the blue-most inactive BPheo transition, $|\Delta\mu|$ changes from 4.2 D/*f* in the open reaction centers to about 2.6 D/*f* when $P^+\Phi_A Q_A^-$ is formed. The other BPheo transition (at 760 nm in open reaction centers) has a $|\Delta\mu|$ of 3.5 D/*f* in the open reaction centers and $|\Delta\mu| = 2.4$ D/*f* when $P^+\Phi_A Q_A^-$ is formed.

Figure 2 shows that when the inactive Pheo band is essentially isolated in the spectrum the same drop in $|\Delta\mu|$ upon $P^+\Phi_A Q_A^-$ formation is observed in the remaining BPheo band. It is therefore excluded that the apparent change in $|\Delta\mu|$ is caused by overlap of the two BPheo absorption bands.

From the above observations we conclude that upon formation of $P^+\Phi_A Q_A^-$ the electrostatic charges of the radical pair induce a change in the permanent dipole moment of the ground and/or excited state of the BPheo molecules. Because the changed permanent dipole moments in general will have a direction different from that of the permanent dipole already present (the

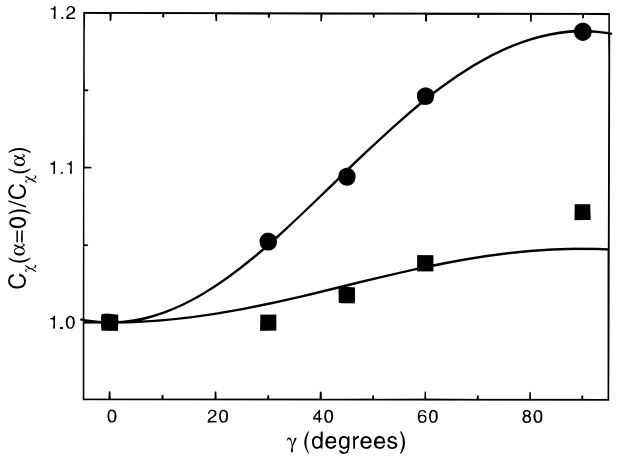

Figure 7. Amplitude of second-derivative Stark amplitude C_χ found for the isolated active BPheo band measured in the Φ_B-exchanged reaction centers of *Rb. sphaeroides* as a function of the angle of polarization of the measuring light beam γ. The polarizer was inserted behind the sample, which was placed at an angle of 45° with the optical axis of the setup. The data points represent the amplitudes resulting from the fit in the open (dots) and charge-separated (squares) reaction centers. The solid lines represent the theoretical dependence for $\zeta = 20°$ and $\zeta = 40°$, respectively.

direction of the change in $|\Delta\mu|$ induced by the charges is determined by the resulting electric field while the permanent dipole is fixed in the molecular frame), the angle between the permanent dipole moment and the (optical) transition dipole of the BPheo should also change when an additional dipole is induced by the charges in the radical pair. To check this we measured the dependence of the Stark intensity on the angle between the externally applied field and the polarization vector of the measuring light by rotating the sample cell until the applied electric field makes a 45° angle with the optical path and inserting a Glan−Thomson polarizer behind the sample.[6] Placing the polarizer *behind* the sample ensures that all spectra are measured under exactly the same conditions, including the same excitation light intensity. The Stark spectra in the closed and charge-separated reaction centers were measured for different angles of the direction of polarization and all fitted using the same procedure as above but letting only the amplitude of the second-derivative Stark contribution vary. (Of course the absorption spectra do not change when the direction of polarization is rotated.) The results of these fits are plotted in Figure 7, which shows that indeed the angle ζ changes upon creation of the radical pair $P^+\Phi_AQ_A^-$. A least-squares fit using eq 2 gives values for ζ of about 20° in open reaction centers and 40° when $P^+\Phi_AQ_A^-$ is formed. Note that the values for $|\Delta\mu|$ in Table 1 were calculated assuming an angle of 10° for both spectra. However, since the differences in apparent $|\Delta\mu|$ for $\zeta = 10°$ and $\zeta = 40°$ are small (about 15%), they do not influence the major conclusions drawn from the table. In view of the uncertainty in the ζ values for glycerol/buffer measurements we did not adjust Table 1 for the angles derived from the fits in Figure 7.

4.2.3. The Difference Dipole Moment Induced by $F_{P+Q_A^-}$. Because in the Stark spectrum of the BPheos no evidence for a contribution of the first-derivative of the absorption is found, the Stark effect in the monomeric pigments region is generally analysed under the assumption that the difference polarizability is very small. As argued in the previous section the difference polarizability is, however, large enough to produce a significant change in $|\Delta\mu|$ when the charge-separated state $P^+\Phi_AQ_A^-$ is formed. The change in dipole moment due to the electrostatic field $\mathbf{F}_{P^+\Phi_AQ_A^-}$, produced by the radical pair, is given by

$$\Delta\mu_{tot} = \Delta\mu_{F=0} + \Delta\mu_{F_{P^+Q_A^-}} = \Delta\mu_{F=0} - \Delta\alpha_{iso}\mathbf{F}_{P^+Q_A^-} \quad (3)$$

where $\Delta\mu_{tot}$ is the total observed difference dipole moment and $\Delta\mu_{F=0}$ is the difference dipole observed in open reaction centers. We have assumed that the difference polarizability $\Delta\alpha_{iso}$ is isotropic so that the induced dipole moment is directed along the inducing field $\mathbf{F}_{P^+Q_A^-}$.

A minimum value for $\Delta\alpha$ can be estimated as follows. When the inducing field is directed more or less (anti-)parallel to the original $|\Delta\mu|$, the change in the difference dipole moment upon $P^+\Phi_AQ_A^-$ formation is equal to the absolute induced dipole moment. In our case this assumption is not strictly true as we find a different angle between the dipole moment and the optical transition dipole (Figure 7) for the two types of reaction centers (open or charge separated). Hence the following calculation yields a lower limit for $\Delta\alpha$. The total measured change in $|\Delta\mu|$ of the inactive BPheo in the open and charge-separated reaction centers is about 1.8 D/*f* (Table 1, 4.2−2.4 D/*f*). Assuming that $\mathbf{F}_{P^+Q_A^-}$ lies along $\Delta\mu_{F=0}$, from eq 3 the polarizability must be at least

$$\Delta\alpha_{iso} \geq \frac{1.8\ \text{D}/f}{|\mathbf{F}_{P^+Q_A^-}|} \quad (4)$$

The field due to the radical pair $P^+\Phi_AQ_A^-$ was estimated by Steffen *et al.* to be 4.5×10^8 V/m at the geometric center of the inactive BPheo in a vacuum, resulting in

$$\Delta\alpha_{iso} \geq 120\ \text{Å}^3\epsilon_{eff} \quad (5)$$

with ϵ_{eff} estimated to be 1.6 in ref 7 or 2.1 in this work. Hence the difference polarizability is at least 190−250 Å^3, depending on the estimated ϵ_{eff}. (Of course ϵ_{eff} should be *at least* 1.)

4.2.4. Implications for the Results of Stark Fits. A simultaneous fit of the Stark effect and absorption spectrum using both a second- and a first-derivative component for the isolated BPheo band in the Φ_B-exchanged reaction centers resulted in a value for $|\Delta\mu|$ that was about 5−10% lower (depending on the fit procedure and starting values) than those reported in Table 1. The resulting value for $Tr(\Delta\alpha)$ is about 220 Å$^3/f^2$, agreeing well with the estimation made from the changes in $|\Delta\mu|$ induced by the radical pair electric field in charge-separated reaction centers (eq 5). It seems therefore reasonable to include a difference polarizability term in the fits, even though the χ^2 of the fit does not appreciably decrease.

4.2.5. BChl Region. In the BChl region our fit is hampered by the strong spectral overlap. Yet, it can be seen from the "native" panels in Table 1 that the changes in the BChl region are much smaller than those for the BPheos and of opposite sign (an increase of $|\Delta\mu|$ upon radical pair formation). This difference in sign can be understood by assuming that the orientations of the optical transition moment, $|\Delta\mu|$, and the polarizability tensor are about the same in BChl and BPheo. Because of the different orientation of the pigment relative to the radical pair electric field, as evidenced by the opposite electrochromic bandshift, one would then expect the changes in $|\Delta\mu|$ to be opposite as well, as observed. The much smaller amplitude of the effect indicates that the polarizability is smaller for the BChl than for BPheo, or that the relative orientation of the difference polarizability tensor is different.

5. Conclusions

The Stark effect spectrum was measured and analyzed in native reaction centers of *Rb. sphaeroides* and in reaction centers

in which the BPheo was selectively replaced by plant Pheo. The Stark effect of the reconstituted Pheo could be accurately described with a second-derivative absorption line shape, much like the native BPheo, indicating that the Stark effect is mainly caused by the difference in permanent dipole moment between the ground and excited state. From the fit results it is concluded that the change in dipole moment $|\Delta\mu|$ is about 1.5 D/*f*, a factor of 2−3 lower than that of the BPheo pigment. This result is in agreement with measurements on isolated pigments in solution.[3,4,20]

By comparing the Stark effect in open and charge-separated reaction centers (state $P^+\Phi_A Q_A^-$), we conclude that especially for BPheo, the permanent dipole moment $|\Delta\mu|$ is influenced by the presence of the radical pair Coulomb field, indicating that the pigment also exhibits a difference in polarizability between the ground and excited states. This difference is estimated to be about 200 Å^3, contrasting with the generally accepted notion, based on the near second-derivative lineshape of the Stark spectrum, that $\Delta\alpha$ is very small. Including such a difference polarizability in the fits changes the value of $|\Delta\mu|$ by 5−10% compared to the fits using only second-derivative contributions to the Stark spectrum.

The observation that the two exchanged Pheos are optically much more alike than the native BPheos is in agreement with the optical parameters of the two pigments and the estimated effective dielectric constant.[7] Also the much smaller electrochromic shift upon $P^+Q_A^-$ formation of Pheo compared to that of BPheo can be explained solely by the difference in electrooptical parameters. We find that due to the substantial difference polarizability of the pigments the method employed in ref 7 for estimating the dielectric screening by the protein matrix needs to be extended (see Appendix).

Finally, a note of caution: From our own work and from the fact that different groups have reported quite different values for the electrooptical parameters of the pigments in photosynthetic reaction centers,[1−3,6,24] we conclude that the results of the methods generally used for analyzing the Stark spectrum (fitting it to a sum of zeroth-, first-, and second-order derivatives of the absorption spectrum) depend heavily on the parametrization of the absorption spectrum and on the exact procedure used for fitting. Drawing conclusions on, for example, the magnitude of a difference polarizability solely from fit results without additional and independent supporting evidence is therefore in our opinion not warranted.

Appendix

Electrochromic Shifts in Polarizable Molecules. Guided by our result that the monomeric pigments in photosynthetic reaction centers undergo a nonnegligible change in permanent dipole moment when placed in an internal electric field, we present an adaptation of the method used by Steffen et al.[7] for estimating the effective dielectric constant in the reaction center, which takes into account the different polarizability of the relevant pigment. The change in the energy of an optical transition, and hence the observed electrochromic bandshift due to an electric field, is equal to

$$\Delta\nu_{obs} = \Delta\mu\, F_{int} \tag{6}$$

where we assume for simplicity that $\Delta\mu$ is a scalar.[25] While this is an approximation, it does provide a lower limit for the polarizability and serves to indicate the line of argumentation. F_{int} is the magnitude of the electric field $\boldsymbol{F}_{int}$ experienced by the pigment.

For a pigment in a vacuum the calculated bandshift is

$$\Delta\nu_{calc} = \Delta\mu\, F_{\epsilon=1} \tag{7}$$

where $F_{\epsilon=1}$ is the magnitude of the field that would be present at the location of the pigment under study if the system were in a vacuum. The two fields are related through the effective dielectric constant ϵ_{eff}, which represents screening by the protein

$$F_{int} = \frac{F_{\epsilon=1}}{\epsilon_{eff}} \tag{8}$$

Hence,

$$\frac{\Delta\nu_{calc}}{\Delta\nu_{obs}} = \epsilon_{eff} \tag{9}$$

When, however, the molecule is polarizable, the difference dipole moment changes when the field is applied:

$$\Delta\mu_{F_{int}} = \Delta\mu_{F_{int}=0} + \Delta\alpha_{iso} F_{int} \tag{10}$$

where we have assumed that the polarizability $\Delta\alpha_{iso}$ is a scalar. Now the observed bandshift equals

$$\Delta\nu_{obs} = \Delta\mu_{F_{int}} F_{int} = [\Delta\mu_{F_{int}=0} + \Delta\alpha_{iso} F_{int}] F_{int} \tag{11}$$

and, using eq 8

$$\Delta\nu_{obs} = \frac{1}{\epsilon_{eff}}\left[\Delta\mu_{F_{int}=0} F_{\epsilon=1} + \Delta\alpha_{iso}\frac{F_{\epsilon=1}^2}{\epsilon_{eff}}\right] \tag{12}$$

Consequently eq 9 becomes

$$\frac{\Delta\nu_{calc}}{\Delta\nu_{obs}} = \frac{\epsilon_{eff}^2}{\epsilon_{eff} + \dfrac{\Delta\alpha_{iso}}{\Delta\mu} F_{\epsilon=1}} \tag{13}$$

It follows that the values reported for the dielectric constant in ref 7 and in this work are only valid for the approximation

$$\frac{\Delta\alpha_{iso}}{\Delta\mu} F_{\epsilon=1} \ll \epsilon_{eff} \tag{14}$$

Using our value for $\Delta\mu$ and the field calculated in ref 7 for $P\Phi Q_{AA}^{+-}$ at the $BPheo_B$ (4.5×10^8 V/m) eq 14 yields

$$\Delta\alpha_{iso} \ll \frac{\Delta\mu}{F_{\epsilon=1}} \ll 280\ \text{Å}^3 \epsilon_{eff} \tag{15}$$

In the main body of this article we estimated that $\Delta\alpha_{iso}$ is *at least* 120 Å$^3\epsilon_{eff}$. We conclude therefore that even the relatively small polarizability of the BPheo molecules is large enough to hamper the estimation of the effective dielectric constant of the surrounding protein when simply comparing the observed bandshift with the experimentally measured shift as in ref 7. Equation 13 shows that this simple procedure actually underestimates the effective dielectric constant. More important, the estimated value of ϵ_{eff} depends on the magnitude of the field that creates the electrochromic shift. This might be one of the reasons why in ref 7 the estimate for ϵ_{eff} is quite different when $P\Phi Q_{AA}^{+-}$, $P^+\Phi_A Q_A$, and $P\Phi Q_{AA}^-$ reaction centers are compared, because in all three cases the magnitude of the field is different.

Another factor that might cause errors in the determination of ϵ_{eff} is the rather crude simplification of assuming that the

dielectric screening is constant over the volume of the charge cloud of the pigment under study and, perhaps even more important, that it is isotropic (see for instance refs 26 and 27). Although the magnitudes of these effects are difficult to assess, it seems unlikely that on a microscopic scale the dielectric screening by the protein matrix is the same in every direction. Clearly, when one compares the field produced by the primary donor cation or by the charged semiquinone, the direction of the two Coulomb fields is quite different, so anisotropic effects are expected.

References and Notes

(1) Lockhart, D. J.; Boxer, S. G. *Biochemistry* **1987**, *26*, 664.

(2) Middendorf, T. R.; Mazzola, L. T.; Lao, K. Q.; Steffen, M. A.; Boxer, S. G. *Biochim. Biophys. Acta* **1993**, *1143*, 223.

(3) Boxer, S. G. in *The Photosynthetic Reaction Center*; edited by Deisenhofer, J., Norris, J. R., Eds.; Academic Press: San Diego, CA, 1993; Vol. 2, p 179.

(4) Gottfried, D. S.; Boxer, S. G. *J. Lumin.* **1992**, *51*, 39.

(5) Scherer, P. O. J.; Fischer, S. F. *Chem. Phys. Lett.* **1986**, *131*, 153.

(6) Lösche, M.; Feher, G.; Okamura, M. Y. *Proc. Natl. Acad. Sci U.S.A.* **1987**, *88*, 7537.

(7) Steffen, M. A.; Lao, K.; Boxer, S. G. *Science* **1994**, *264*, 810.

(8) Meyer, M.; Scheer, H. *Photosynth. Res.* **1995**, *44*, 55.

(9) Franken, E. M.; Shkuropatov, A. Y.; Francke, C.; Neerken, S.; Gast, P.; Shuvalov, V. A.; Hoff, A. J.; Aartsma, T. J. *Biochim. Biophys. Acta* **1997**, *1319*, 242.

(10) Bolton, J. R.; Clayton, R. K.; Reed, D. W. *Photochem. Photobiol.* **1969**, *9*, 209.

(11) Knapp, E. U.; Scherer, S. F.; Fischer, P. O. J.; *Biochim. Biophys. Acta* **1986**, *852*, 295.

(12) Lous, E. J.; Hoff, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6147.

(13) Vrieze, J.; Hoff, A. J. *Biochim. Biophys. Acta* **1996**, *1276*, 210.

(14) van Dijk, B.; Nozawa, T.; Hoff, A. J. *Spectrochim. Acta A* **1997**, *54*, 1269.

(15) Kleinfeld, D.; Okamura, M. Y.; Feher, G. *Biochemistry* **1986**, *23*, 5780.

(16) Feher, G.; *Photochem. Photobiol.* **1971**, *14*, 373.

(17) Jankowiak, R.; Small, G. J. In *The Photosynthetic Reaction Center*; Deisenhofer, J.; Norris, J. R., Eds.; Academic Press: San Diego, CA, 1993; Vol. 2, p 133.

(18) Hayes, J. M.; Small, G. J. *J. Phys. Chem.* **1986**, *90*, 4928.

(19) van Stokkum, I. H. M.; Scherer, T.; Brouwer, A. M.; Verhoeven, J. W. *J. Phys. Chem.* **1994**, *98*, 852.

(20) Krawczyk, S. *Biochim. Biophys. Acta* **1991**, *1056*, 64.

(21) f is the local field correction factor that correlates the field experienced by the pigment with the externally applied field: $F_{ext} = fF_{int}$.

(22) Not surprisingly because the active pigment is of course much closer to Q_A.

(23) We have assumed that the angle between $\Delta\mu$ and the optical transition moment is practically equal for BPheo and Pheo. Assuming a similar orientation of these molecules in the reaction center one finds identical angles between $|\Delta\mu|$ and $\boldsymbol{F}_{P^+\Phi_A Q_A^-}$ for BPheo and Pheo.

(24) Lockhart, D. J.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 107.

(25) Since we are dealing with *internal* fields the angle between the field and the dipole moment is the same in each reaction center. Therefore, taking $\Delta\mu$ as a scalar effectively corresponds to assuming that $\boldsymbol{F}_{int}$ lies along $\Delta\mu$.

(26) Pollock, E. L.; Alder, B. J.; Pratt, L. R. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 49.

(27) Zeng, C.; Davis, M. E.; McCammon, J. A. *Chem. Phys. Lett.* **1990**, *173*, 246.