Two Distinct Conformations of the Primary Electron Donor in Reaction Centers from *Rhodobacter sphaeroides* Revealed by ENDOR/TRIPLE-Spectroscopy†

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ABSTRACT: The effect of solubilization of photosynthetic reaction centers (RCs) from *Rhodobacter sphaeroides* with different detergents on the electronic structure of the oxidized primary donor, $P^{\bullet+}$, is investigated. Electron paramagnetic resonance spectroscopy and related multiple resonance techniques (ENDOR/Special TRIPLE) show that two distinct conformations of $P^{\bullet+}$ can be obtained, depending on the detergent properties, the detergent/RC ratio, and the temperature. The two states correspond to different positions of the long-wavelength Q_y-band of the neutral state, P ($\lambda_1 = 866$ nm and $\lambda_2 = 850$ nm at room temperature) and therefore are called $P_{866}^{\bullet+}$ and $P_{850}^{\bullet+}$, respectively. $P_{866}^{\bullet+}$ is found in chromatophores and in RCs solubilized with nonionic detergents and bile salts. $P_{850}^{\bullet+}$ is induced by zwitterionic and ionic detergents with aliphatic hydrophobic chains. The TRIPLE resonance spectra reveal that both states coexist in the range $\lambda_2 < \lambda_{max} < \lambda_1$. The main property of the detergent that determines the ability to induce $P_{850}^{\bullet+}$ is the polarity of the head group. A simple phenomenological model is presented that relates the standard Gibbs free energy difference between the two conformations to the detergent/RC ratio and the temperature. Of special interest is the observation that the widely used detergent LDAO can induce $P_{850}^{\bullet+}$ upon freezing the RCs without cryoprotectants. The spectroscopic properties of the two states are compared and their possible roles in RC function are discussed.

The primary photochemical reactions of bacterial photosynthesis occur in a specialized membrane-bound pigment−protein complex, called the reaction center (RC[1]). In the last decade, high-resolution X-ray structures of RCs from two different species of purple nonsulfur bacteria, i.e., *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* [reviewed by Lancaster et al. (1995)], became available. Protocols were developed to manipulate the RC structure either chemically (Scheer & Struck, 1993) or through site-directed mutagenesis [for a recent review, see Williams and Taguchi (1995)]. Owing to these advances, the bacterial RC has become the ideal system for the elucidation of electron transfer mechanisms in proteins.

Light absorption by the RC results in the formation of the excited singlet state of the primary electron donor P, an excitonically coupled dimer of bacteriochlorophylls. Within ∼4 ps, an electron is transferred from P via a monomeric BChl to a BPh, and subsequently in about 200 ps to one of two quinone acceptors, Q_A. Finally a stable charge separated state, $P^{\bullet+}Q_B^{\bullet-}$, is formed in ∼0.1 ms (Feher et al., 1989; Zinth & Kaiser, 1993; Woodbury & Allen, 1995).

Studies of RCs in membrane fragments (chromatophores) using optical techniques are hampered by the presence of LH pigments and therefore are most often performed on isolated, detergent solubilized RC complexes. It has been noticed very early that the optical and photochemical properties of isolated RCs depend on experimental conditions. In particular, the exact peak position of the long-wavelength near-IR absorption (Q_y-band) of P varies by up to 15 nm (Clayton, 1978; Debus et al., 1985). Wang et al. (1994) observed that in purified RCs from different species of BChl *a*-containing purple bacteria the Q_y-band of P is located either at ∼865 or ∼850 nm, if the RCs are solubilized with the commonly used detergent *N*-lauryl-*N*,*N*-dimethylamine-*N*-oxide (LDAO). However, it is possible to convert the RCs from one state to the other by addition of certain detergents (Wang et al., 1994). Recently, it was demonstrated that removal of the detergent also causes a blueshift of the Q_y-band of P down to 846 nm in RCs from *R. sphaeroides* (Gast et al., 1996).

One of the most sensitive probes for small changes of the pigment−protein interactions in RCs is the electronic structure of the cation radical of the primary donor, $P^{\bullet+}$. The reason for this sensitivity is that the distribution of the unpaired electron within the dimer strongly depends on the electronic coupling and geometry of the two BChl moieties as well as the interactions with single amino acid residues (Plato et al., 1992; Rautter et al., 1994, 1995). The electronic structure of $P^{\bullet+}$ can be obtained by determining the electron-nuclear hfcs with EPR methods like ENDOR or TRIPLE. The assignment of the hfcs to specific molecular positions then yields a map of the spin density distribution of the

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[1] Abbreviations and symbols: α, Coulomb integral; β, resonance integral; ϵ, extinction coefficient; ζ, detergent/RC ratio in units of 10^3; λ, wavelength; ν, frequency; ρ, spin density; *A*, hyperfine coupling constant; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; *c*, concentration; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; *G*, Gibbs free energy; hfc, hyperfine coupling; HOMO, highest occupied molecular orbital; IR, infrared; *K*, equilibrium constant; LH, light harvesting complex; MO, molecular orbital; OD, optical density; P, primary electron donor; $Q_{A,B}$, quinones in the RC; $Q_{x,y}$, optical transitions in BChls; *R*, ideal gas constant; RC, reaction center; rf, radio frequency; *T*, temperature; TRIPLE, electron-nuclear-nuclear triple resonance; *X*, mole fraction.

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unpaired valence electron in the molecule under investigation [for reviews, see Möbius et al. (1989), Lubitz (1991), Lubitz & Lendzian (1996)]. The great advantage of these techniques is the possibility to selectively observe the radical ions formed in the charge separation process. Hence, the experiments are not perturbed by the presence of LH complexes and can also be performed on intact chromatophores (Feher et al., 1975; Norris et al., 1975; Rautter et al., 1994). Solubilized RCs are used in ENDOR experiments solely because of the higher resolution obtained in the spectra. This is due to the faster tumbling of these complexes in liquid solution which leads to an averaging of hyperfine anisotropies (Lendzian et al., 1981; Rautter et al., 1994). The most extensive ENDOR investigations of $P^{\bullet+}$ in *R. sphaeroides* have been performed by Lendzian et al. (1993) on RC single crystals and revealed an unequal distribution of the π-spin density in the dimer with a ratio of approximately 2:1 in favor of P_L, i.e., the dimer half that is associated with the L-subunit of the RC-protein. The same spin density distribution is found for $P^{\bullet+}$ in chromatophores from several purple bacterial species that contain BChl *a* (Rautter et al., 1994). However, the situation is different for isolated RCs. In addition to the conformation observed in chromatophores, a second distinct state of the primary donor can be stabilized in detergent-solubilized complexes, which shows a stronger asymmetry of the spin density distribution (Rautter et al., 1994; Käss et al., 1995; Lubitz et al., 1995). Interestingly, the appearance of the second conformation is correlated with a ~15-nm blueshift of the Q_y-band of P (Rautter et al., 1994; Wang et al., 1994; Müh et al., 1996). In addition to the ENDOR results there is experimental evidence for two forms of P in RCs of *R. sphaeroides* (Lous & Hoff, 1986) and *R. viridis* (Gast et al., 1995) from magneto-optical difference spectroscopy. In the case of *R. viridis* even two long-wavelength absorption bands are observed at 4.2 K (Verméglio & Paillotin, 1982). Two RC conformations were also considered by Parot et al. (1987) to explain the complex kinetics of charge recombination in RCs of *R. sphaeroides* and *Rhodospirillum rubrum* at low temperatures measured near 800 nm.

In this paper, we present further evidence that solubilized RCs from *R. sphaeroides* exhibit two distinct conformations of the primary donor, depending on detergent properties and experimental conditions. In particular, we demonstrate that an equilibrium between the two conformations exists that can be shifted by changing the detergent/RC ratio and the temperature. The nature of both states of P and their possible relevance to RC function are discussed.

MATERIALS AND METHODS

RCs from photoheterotrophically grown cells of *R. sphaeroides* 2.4.1 were prepared according to Buchanan et al. (1993) with slight modifications. The buffer used throughout (TE) contained 10 mM Tris/Cl (pH 8.0) and 1 mM EDTA. Isolated RCs were finally solubilized in 0.1% (4.3 mM) LDAO. For detergent exchange, a stock solution of the respective detergent in TE was added to the RC samples and the LDAO was removed by repeated dialysis. Samples were reconcentrated to $OD_{805} \approx 100$ by spinning in centricon 30 tubes (Amicon). The following detergents were tested. (1) nonionic: *n*-alkylpolyoxyethylene ethers (Brij series: $C_{12}E_{10}$, $C_{16}E_{10}$, $C_{16}E_{20}$, $C_{18}E_{20}$, $C_{18:1}E_{20}$; Sigma), *tert*-octylphenyl polyoxyethylene ether (Triton X-100; Fluka Biochemika), ethylphenyl polyoxyethylene ether (Nonidet P-40; Boehringer), polyoxyethylenesorbitan monolaurate (Tween 20; Boehringer), *n*-octyl-β-D-glucopyranoside (β-OG; Boehringer), and *n*-dodecyl-β-D-maltoside (β-DM; Boehringer). (2) zwitterionic: *N*-(*n*-alkyl)-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonates (sulfobetaines: SB8, SB10, SB12, SB14, SB16; Sigma), and *N*-(3-Cholamidopropyl)-*N*,*N*-dimethyl-3-ammonio-1-propane sulfonate (CHAPS; Boehringer). (3) ionic: sodium cholate (Boehringer), sodium deoxycholate (DOC; Merck), and *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB; Fluka Chemika).

Optical spectra were recorded using a Cary 05E spectrophotometer (Varian) equipped with a thermostated sample holder of local design. $P^{\bullet+}$ was generated by cross illumination of the samples with red light ($\lambda > 600$ nm) from a Schott KL 1500 light source via an 8-mm light guide. The extinction coefficient used for the determination of RC concentrations was $\epsilon_{805} = 288\ mM^{-1}\ cm^{-1}$ (Straley et al., 1973).[2]

The EPR and ENDOR experiments were performed by using a Bruker ESP 300E spectrometer equipped with home-built ENDOR/TRIPLE accessories and a Bruker nitrogen gas cooling system [for details, see Rautter et al. (1994)]. The EPR measurements were carried out in a standard rectangular cavity (Bruker ER 4102 ST). For the ENDOR and TRIPLE experiments a TM_{110} cavity of local design similar to the one described by Zweygart et al. (1994) was used. For the experiments in liquid solution ($T = 278-298$ K), $P^{\bullet+}$ was generated by continuous illumination of the sample inside the cavity with actinic light. Experiments on $P^{\bullet+}$ in RCs in frozen solution or in chromatophores ($T = 160$ K) were carried out by illuminating the samples for ~30 s at ambient temperatures prior to freezing in liquid nitrogen. Illumination was continued during the measurements.

RESULTS

In order to evaluate the conditions for the occurrence of the second conformation of P, we have tested the effects of various commercially available detergents on solubilized RCs from *R. sphaeroides* 2.4.1. Among these (see Materials and Methods), only the cationic detergent CTAB [cf. Wang et al. (1994)] and the zwitterionic sulfobetaines induce a pronounced blueshift of the ground state absorption maximum, λ_{max}, of the long-wavelength Q_y-band of P. In all other cases λ_{max} is located at 866 ± 2 nm at room temperature. The magnitude of the induced blueshift for a given detergent depends on the detergent/RC ratio as well as on the temperature. At room temperature, λ_{max} varies between 850 and 862 nm for the sulfobetaines.[3] LDAO is also able to induce a blueshift of λ_{max}, but at much higher detergent/RC ratios than the sulfobetaines (Müh et al. 1996). Usually $\lambda_{max} = 865 \pm 1$ nm is found in LDAO at room temperature. Figure 1 shows the comparison of the near-IR region of the absorption spectra of RCs from *R. sphaeroides* 2.4.1 solubilized with 4.3 mM LDAO and 12.5 mM SB12, respectively, which represent the two extreme cases. The blueshift of λ_{max} to 850 nm is correlated with a decrease of the

[2] Straley et al. (1973) actually determined $\epsilon_{802} = 288 \pm 14\ mM^{-1}\ cm^{-1}$ for strain R26.1. We used this value for the 805-nm band of RCs from strain 2.4.1.

[3] In the case of CTAB, we were not able to determine the whole range of λ_{max}, since this detergent caused denaturation of the RCs.

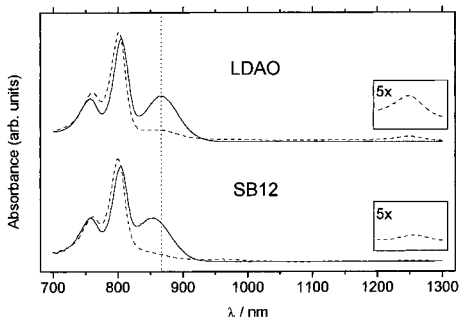

FIGURE 1: Near-IR absorption spectra of dark-adapted (solid) and illuminated (dashed) RCs from *R. sphaeroides* 2.4.1 (c_{RC} = 1.5 μM) solubilized with either 4.3 mM LDAO (λ_{max} = 866 nm, see vertical dotted line) or 12.5 mM SB12 (λ_{max} = 850 nm) at T = 298 K. The inserts show the characteristic absorption band of the cation radical $P^{\bullet+}$ at 1250 nm (scaled up 5-fold).

oscillator strength of the 805-nm band by ~20% and that of the characteristic absorption band of the cation radical of P near 1250 nm (insets to Figure 1) by ~60%. These observations are in good agreement with the results obtained by Wang et al. (1994) for RCs from various species of purple bacteria.

The Special TRIPLE spectra of the primary donor cation radical, $P^{\bullet+}$, in samples exhibiting the two extreme positions of λ_{max} are shown in Figure 2 (top and bottom, respectively). The spectra clearly show that the different absorption maxima of P are correlated with two distinct spectroscopic species of $P^{\bullet+}$. These two conformations of the primary donor are referred to in the following as $P_{866}^{\bullet+}$ and $P_{850}^{\bullet+}$, respectively. This labeling is in accordance with the usual nomenclature of primary donor pigments in photosynthesis and refers to the positions of the Q_y absorption maxima at room temperature. The hfcs (Table 1) were obtained by using a spectral deconvolution program for the evaluation of overlapping lines (Tränkle & Lendzian, 1989).

The state stabilized by LDAO at room temperature, $P_{866}^{\bullet+}$, is also obtained by illumination of chromatophores with actinic light (*vide infra*). Therefore, this state represents the conformation of the oxidized primary donor that is found in RCs embedded in their natural membrane environment[4] (Rautter et al. 1994). The EPR signal of $P_{866}^{\bullet+}$ is narrower (ΔB_{pp} = 0.96 ± 0.02 mT) than that of monomeric BChl $a^{\bullet+}$ *in vitro* (ΔB_{pp} = 1.40 ± 0.02 mT) because the unpaired spin density is spread over both dimer halves [cf. Figure 17 in Lendzian et al. (1993)]. $P_{866}^{\bullet+}$ has also been observed in the ENDOR experiments on RC single crystals which allowed the unambigous assignment of the hfcs to specific molecular positions on P_L and P_M (Gessner et al., 1992; Lendzian et al., 1993). The most prominent hfcs arise from the β protons at positions 3, 4, 7, and 8 of rings II and IV and from the methyl protons at positions 1a and 5a of rings I and III (for numbering, see Figure 3). The detailed analysis of the spectra of $P_{866}^{\bullet+}$ yields the asymmetric spin density distribution of approximately 2:1 in favor of P_L (Gessner et al., 1992; Lendzian et al., 1993; Rautter et al., 1994).[5] In all other cases where $\lambda_{max} \approx$ 866 nm, i.e., for RCs solubilized with nonionic detergents or bile salts, the EPR and ENDOR

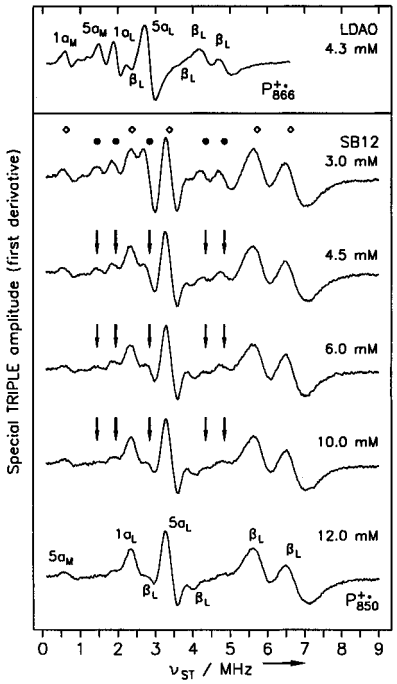

FIGURE 2: Comparison of the ^{1}H-Special TRIPLE spectra in liquid solution of light-induced $P^{\bullet+}$ in RCs from *R. sphaeroides* 2.4.1 solubilized with either LDAO or SB12 at T = 288 K. The spectra at the top and the bottom correspond to RCs exhibiting the two extreme positions of the Q_y-band of P at 866 and 850 nm, respectively. All hyperfine couplings assigned to P_L are larger in $P_{850}^{\bullet+}$ than in $P_{866}^{\bullet+}$, and *vice versa* for P_M. For the assignment of signals to molecular positions, see text and Figure 3. The detergent concentrations given are those of the dialysis buffer used for detergent exchange (cf. Materials and Methods). For the spectra representing mixtures of states the line positions belonging to the two different conformations (cf. Table 1) are marked with full circles ($P_{866}^{\bullet+}$) and open diamonds ($P_{850}^{\bullet+}$), respectively. The arrows indicate the decrease of the fraction of $P_{866}^{\bullet+}$ with increasing SB12 concentration. Experimental conditions: microwave power 16 mW, rf power 2 × 150 W, rf modulation depths 200 kHz (frequency 12.5 kHz), accumulation time 2 h.

spectra of light-induced $P^{\bullet+}$ (not shown) are also characteristic for this state.[6]

The other extreme case with $\lambda_{max} \approx$ 850 nm, which corresponds to the $P_{850}^{\bullet+}$ state, is characterized by an EPR linewidth ΔB_{pp} of 1.12 ± 0.02 mT. This value is closer to that of monomeric BChl $a^{\bullet+}$ (Table 1), indicating a more asymmetric spin density distribution within the dimer in $P_{850}^{\bullet+}$. The enhanced asymmetry is manifested by increased magnitudes of hfcs assigned to protons of P_L and a decrease of those assigned to P_M in the corresponding TRIPLE spectrum (Figure 2, bottom). The assignment of methyl proton hfcs is based on the assumption that the characteristic ratio A(5a)/A(1a) of each BChl moiety (Plato et al., 1988; Feher, 1992) is conserved in $P_{850}^{\bullet+}$ and $P_{866}^{\bullet+}$ (Table 1), in

[4] $P_{866}^{\bullet+}$ is also found by ENDOR spectroscopy of antenna-deficient chromatophores of *R. sphaeroides* (Rautter et al., unpublished). Optical spectra of such chromatophores show λ_{max} = 866 nm (Beekman et al., 1995).

[5] A small overall shift of the spin density (3%) toward P_L is observed for strain 2.4.1 as compared with R26.1, which was attributed to the presence of the carotenoid in the former species (Gessner et al., 1992). The spin density asymmetry of 2:1 in favor of P_L is inferred from the methyl proton hfcs. Analysis of the β-proton hfcs yields 1.5:1 to 1.6:1 in favor of P_L, depending on the chosen assignment, which is not unambiguous for the β-protons (Lendzian et al., 1993).

[6] A small fraction of $P_{850}^{\bullet+}$ seems to be present in liquid solution samples of RCs solubilized with Brij 36 T ($C_{12}E_{10}$) as judged from signals in the Special TRIPLE spectra that are characteristic for the β-protons. However, this cannot be detected in the corresponding ENDOR spectra in frozen solution.

Table 1: ^{1}H-hfcs (MHz), Ratios of hfcs and EPR Linewidths of the Two States of $P^{\bullet+}$ in Liquid RC Solution of *R. sphaeroides* Compared with Data of Monomeric BChl $a^{\bullet+}$ *in vitro* and in the Mutant HL(M202)

	R. sphaeroides			
	$P_{866}^{\bullet+}$ [b]	$P_{850}^{\bullet+}$ [c]	$P_L^{\bullet+}$ in HL(M202) [d]	BChl $a^{\bullet+}$ [e] *in vitro*
$A(5a_L)$ [a,f]	5.70	6.86	7.44	
$A(1a_L)$	3.96	5.04	5.75	9.50 (5a)
$A(5a_M)$	3.20	1.50		4.85 (1a)
$A(1a_M)$	1.37	0.65[g]		
$A(\beta_L)$ [a,h]	9.70	13.50	13.70	16.43 (4)
	8.70	11.80	12.45	13.59 (3)
	6.25	7.75	8.10	13.00 (7)
	4.50	5.70	6.25	11.61 (8)
$[A(5a)/A(1a)]_L$ [i]	1.44	1.36	1.29	1.90
$[A(5a)/A(1a)]_M$ [i]	2.34	2.31		
$\sum A(CH_3)$ [j]	14.23	14.05	13.19	14.35
ρ_L/ρ_M [k]	2.11	5.53		
ΔB_{pp}/mT (exp) [l]	0.96	1.12	1.22	1.40
ΔB_{pp}/mT (sim) [l]	0.96	1.12	1.22	1.40

[a] All hfcs were obtained from liquid solution spectra unless stated otherwise; errors of methyl hfcs ±30 kHz and of β hfcs ±50 kHz. [b] The state $P_{866}^{\bullet+}$ is observed in RCs solubilized with nonionic detergents, bile salts, and low LDAO/RC-ratios (see text). [c] The state $P_{850}^{\bullet+}$ is observed in RCs solubilized with the cationic detergent CTAB and the zwitterionic sulfobetaines (see text). [d] Values from Rautter et al. (1995); see also Huber et al. (1996). In the mutant HL(M202) P_M is replaced by a BPh. [e] Values from Lubitz et al. (1984). The assignments to molecular positions are given in parentheses (see Figure 3). [f] For the numbering of molecular positions see Figure 3; for the assignment see text. [g] This hfc was obtained from frozen solution spectra (not shown), error ±100 kHz. [h] β_L proton hfcs (positions 3, 4, 7, and 8 in Figure 3); for the assignment see text. [i] Ratio of methyl proton hfcs in positions 5a and 1a on P_L and P_M, respectively (see text). [j] Sum of all methyl proton hfcs. [k] Ratio of spin densities on P_L and P_M as determined from methyl proton hfcs in positions 1a and 5a; $\rho_L/\rho_M = \sum A(CH_3)_L/\sum A(CH_3)_M$. [l] Experimental and simulated peak-to-peak Gaussian envelope EPR linewidth (spectra not shown); experimental error ±0.02 mT.

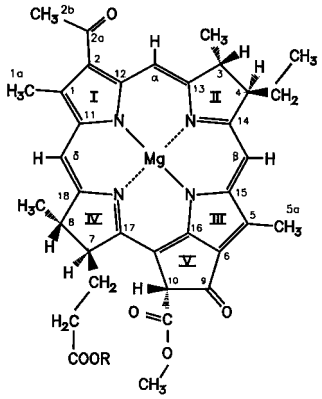

FIGURE 3: Structure of BChl *a* with the numbering scheme (R = phytyl).

agreement with earlier studies (Rautter et al., 1992, 1994). This assignment was recently corroborated by ENDOR experiments on $P_{850}^{\bullet+}$ in frozen, LDAO-containing RC single crystals (Lubitz et al., unpublished) and yields an asymmetric spin density distribution of approximately 5.5:1 in favor of P_L. Furthermore the TRIPLE spectrum of $P_{850}^{\bullet+}$ is quite similar to that of $P^{\bullet+}$ in the heterodimer mutant HL(M202) of *R. sphaeroides*. In this mutant, P_M is replaced by a BPh and the unpaired electron of $P^{\bullet+}$ is completely localized on P_L (Rautter et al., 1995; Huber et al., 1996). However, the

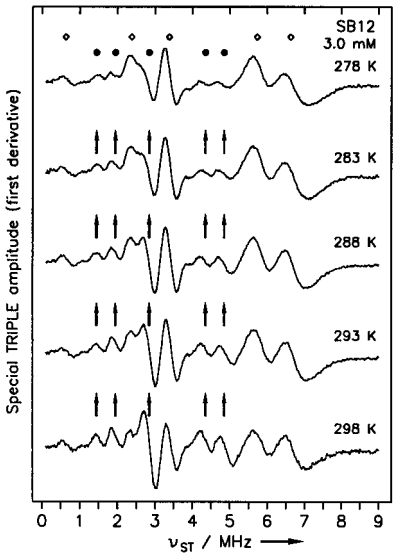

FIGURE 4: Temperature dependence of the ^{1}H-Special TRIPLE spectrum in liquid solution of light-induced $P^{\bullet+}$ in RCs from *R. sphaeroides* 2.4.1 solubilized with 3.0 mM SB12. The line positions belonging to the two different conformations (cf. Table 1) are marked with full circles ($P_{866}^{\bullet+}$) and open diamonds ($P_{850}^{\bullet+}$), respectively. The arrows indicate the increase of the fraction of $P_{866}^{\bullet+}$ with increasing temperature. Experimental conditions see Figure 2.

hfcs of $P_{850}^{\bullet+}$ assigned to protons of P_L are all smaller than those of $P_L^{\bullet+}$ in the mutant (Table 1), indicating that the electron is not confined to one cofactor in the $P_{850}^{\bullet+}$ state.

The dependence of λ_{max} on the detergent/RC ratio raises the question of whether other states besides P_{850} and P_{866} exist or only mixtures of both are present for 850 nm < λ_{max} < 866 nm. We therefore performed EPR and TRIPLE measurements on samples with different SB12/RC ratios. The samples were prepared by varying the SB12 concentration in the dialysis buffer (see Materials and Methods), while keeping all other conditions constant. The corresponding series of Special TRIPLE spectra is shown in Figure 2 together with the two extreme cases. The hfcs that can be identified are characteristic for either $P_{850}^{\bullet+}$ or $P_{866}^{\bullet+}$. Two spectroscopic species are sufficient to explain the spectra. No additional species can be detected. Furthermore, the spectra clearly show that the signals assigned to $P_{866}^{\bullet+}$ are decreased with respect to those of $P_{850}^{\bullet+}$ upon increasing the SB12 concentration. The measured linewidths ΔB_{pp} of the corresponding EPR-spectra (not shown) increase with increasing SB12 concentration from 1.05 to 1.12 ± 0.02 mT. A rough estimate of the mole fraction, *X*, of $P_{850}^{\bullet+}$ can be obtained with the assumption that ΔB_{pp} is a sigmoidal function of the SB12 concentration by analogy with the behavior of λ_{max} in optical titration experiments [cf. Figure 3 in Müh et al. (1996)]. Using the limiting values ΔB_1 = 0.96 mT for $P_{866}^{\bullet+}$ and ΔB_2 = 1.12 mT for $P_{850}^{\bullet+}$, we determined *X* to be approximately 0.5, 0.6, 0.7, 0.8, and 1.0, respectively, with increasing SB12 concentration in Figure 2 ($X \approx 0$ for the LDAO sample).

Figure 4 shows the TRIPLE spectra of the sample with the lowest SB12 concentration recorded at five different temperatures. The signals assigned to $P_{866}^{\bullet+}$ are increased with respect to those assigned to $P_{850}^{\bullet+}$ upon raising the temperature from 278 to 298 K. This trend is reflected by the EPR linewidths, which are decreased correspondingly. We estimated *X* to be 0.60, 0.55, 0.50, 0.45, and 0.40,

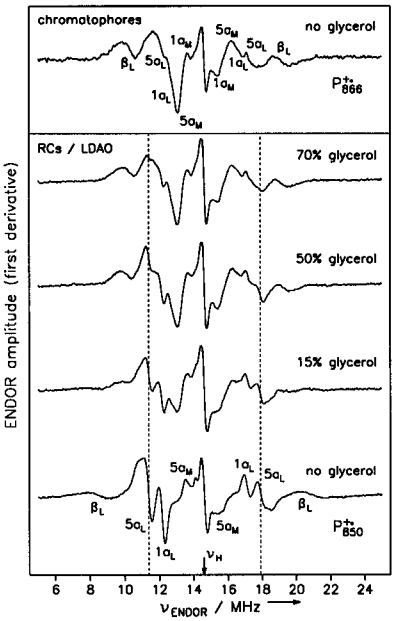

FIGURE 5: ^{1}H-ENDOR spectra in frozen solution of light-induced $P^{\bullet+}$ in RCs from *R. sphaeroides* 2.4.1 solubilized with 0.1% (w/v) LDAO (bottom) and with different amounts of glycerol added as cryoprotectant (in percent volume/volume). The upper trace shows $P_{866}^{\bullet+}$ generated in chromatophores. For the assignment of signals see Figure 3. In the spectra of the glycerol-containing samples the position of one pair of signals that is indicative for the presence of $P_{850}^{\bullet+}$ (methyl proton hfc of position $5a_L$) is marked with dashed lines. Experimental conditions: $T = 160$ K, microwave power 16 mW, rf power 200 W, rf modulation depths 200 kHz (frequency 12.5 kHz), accumulation time 1 h.

respectively, with increasing temperature. The values indicate that for this sample the state $P_{850}^{\bullet+}$ is preferred at 278 K, while $P_{866}^{\bullet+}$ is more stable at 298 K.

LDAO is also able to induce the P_{850} state. Although the conversion of RCs to P_{850} is difficult with LDAO at room temperature (Müh et al., 1996), this can be achieved by freezing LDAO-containing samples (as well as RC single crystals; Lubitz et al., unpublished) without the addition of cryoprotectants. The ENDOR spectrum obtained at 160 K (Figure 5, bottom) is similar to that of $P_{850}^{\bullet+}$ observed with sulfobetaines in frozen solution (not shown). The formation of $P_{850}^{\bullet+}$ can be avoided by addition of glycerol. However, the fraction of $P_{850}^{\bullet+}$ induced by freezing depends on the amount of glycerol added (Figure 5). In the spectra representing mixtures of both conformations, the line pair assigned to position $5a_L$ of $P_{850}^{\bullet+}$ (indicated by dashed lines in Figure 5) is the best indicator for the presence of this state. Note that even with 70% (v/v) glycerol, traces of $P_{850}^{\bullet+}$ can be detected in the ENDOR spectrum, whereas this is not seen in the light-induced $P^{\bullet+}$ spectrum obtained with chromatophores (Figure 5, top). In some samples the induction of P_{850} by freezing seems to be at least partially irreversible, since the dimer band is observed at $\lambda_{max} = 860-864$ nm after thawing. In these cases storage of the samples at 278 K for days did not lead to a recovery of the band at $\lambda_{max} = 866$ nm.

DISCUSSION

In the present study we performed EPR, ENDOR, and Special TRIPLE measurements to characterize detergent effects on bacterial RCs by using the oxidized primary donor $P^{\bullet+}$ as probe. The motivation for such a study comes from

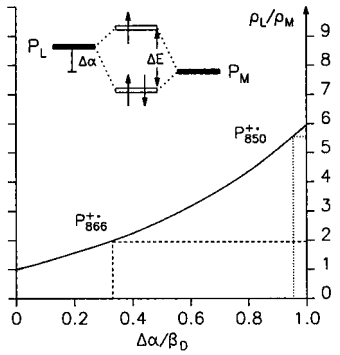

FIGURE 6: Theoretical dependence of the spin density ratio ρ_L/ρ_M of $P^{\bullet+}$ on the ratio of the Coulomb to the resonance integral in the dimer, $\Delta\alpha/\beta_D$ (for details see Plato et al., 1992). The interpretation of the corresponding Special TRIPLE spectra (see text and Figure 2) indicates that the difference between the two distinct states $P_{850}^{\bullet+}$ and $P_{866}^{\bullet+}$ (indicated by dotted and dashed lines, respectively) is mainly due to a change of β_D. Inset: MO scheme describing the interaction of the HOMOs of P_L and P_M in the oxidized state $P^{\bullet+}$ of the primary donor; $\Delta\alpha$ is the energy difference between the monomer orbitals and ΔE that of the dimer orbitals, see eq 1.

the observation that the position of the maximum of the long-wavelength near-IR absorption of P, λ_{max}, varies between 850 and 866 nm in BChl *a*-containing RCs, depending on experimental conditions (Clayton, 1976; Debus et al., 1985; Wang et al., 1994). In earlier studies we found a correlation between these changes and the occurrence of certain hfcs observed in ENDOR experiments of light-induced $P^{\bullet+}$ (Rautter et al., 1994; Lubitz et al., 1995; Müh et al., 1996). The present study clearly demonstrates that only zwitterionic and ionic detergents are able to induce these changes, while RCs solubilized with nonionic detergents have the same spectroscopic properties as those embedded in chromatophores.[6] LDAO and the bile salts represent special cases.

The two spectroscopic forms of $P^{\bullet+}$ that correspond to the two extreme values of λ_{max} differ in the extent of delocalization of the unpaired spin density and hence their EPR linewidths and hfcs. From the assignment of the proton hfcs we can infer that the ratio of the spin densities on P_L and P_M is $\rho_L/\rho_M \approx 2$ in $P_{866}^{\bullet+}$ and $\rho_L/\rho_M \approx 5.5$ in $P_{850}^{\bullet+}$. This stronger localization of the electron on P_L in $P_{850}^{\bullet+}$ can be explained by using a simple MO-model that considers the interaction of the frontier orbitals of the two BChl moieties (Plato et al., 1992; Lendzian et al., 1993). Because of different interactions with the protein environment, the Coulomb energy of the HOMO of P_L is higher than that of P_M, the difference being denoted by $\Delta\alpha$ (Figure 6). The interaction of the two HOMOs, described by the resonance integral β_D, then yields two orbitals of the dimer with an energy difference given by

$$\Delta E = \sqrt{(\Delta\alpha)^2 + (2\beta_D)^2} \qquad (1)$$

In the oxidized state the unpaired electron resides in the upper one of the two dimer orbitals, which has a larger contribution from P_L than from P_M. Consequently, the spin density is also larger on P_L. Since the ratio of spin densities ρ_L/ρ_M is a function of $\Delta\alpha/\beta_D$ (Plato et al., 1992), the observed asymmetry enhancement in $P_{850}^{\bullet+}$ with respect to $P_{866}^{\bullet+}$ can be achieved both by an increase of $\Delta\alpha$ and/or by a decrease of β_D (Rautter et al., 1994). MO calculations (RHF INDO/SP) predict that a change of $\Delta\alpha$ can for example be obtained by changing the hydrogen-bonding pattern to the 2-acetyl

and 9-keto groups of the BChls and/or rotating the 2-acetyl group with respect to the plane of the macrocycle (Plato et al., 1986, 1992). Charges in the vicinity of the macrocycles also influence the Coulomb energies (Plato et al., 1988). In wild-type RCs from *R. sphaeroides*, only one hydrogen bond to P exists between histidine L168 and the 2-acetyl group of P_L. Cleavage of this H-bond causes a symmetrization of the electronic structure of $P^{\bullet+}$, i.e., $\rho_L/\rho_M = 0.76$ in the mutant HF(L168) (Rautter et al., 1995). Moreover, a rotation of the acetyl group would affect the ratio $A(5a)/A(1a)$ of the respective BChl molecule more strongly than observed here (Käss, 1995; Rautter, 1995). The same is true for a change of the ring puckering (Feher, 1992). In contrast, a decrease of β_D can simply be achieved by increasing the distance and/or changing the angle between the two macrocycles without major impact on either the molecular geometry or the orientation of the acetyl groups. We therefore think that the difference between P_{850} and P_{866} is mainly due to a change of β_D. In particular, the hydrogen-bonding pattern should be the same in both states. However, a change of $\Delta\alpha$ due to reorientation of nearby polar side chains or of the protein backbone cannot be excluded. $\Delta\alpha$ could also be changed due to long-range electrostatic effects of amino acid residues in outer parts of the RC protein that act differently on $P_L^{\bullet+}$ and $P_M^{\bullet+}$.

Further insights can be gained by considering the optical properties of $P^{\bullet+}$. Parson et al. (1992) analyzed the transition at 1250 nm in RCs from *R. sphaeroides*. Their MO treatment shows that the oscillator strength of this band is strongly affected by $\Delta\alpha$, while the line position remains constant. More recently, Reimers and Hush (1995a,b) have suggested that the absorption band at 1250 nm is due to a triplet-coupled transition that is located on P_M and actually spin-forbidden. The state $P^{\bullet+}$ is modeled by a monomeric cation $P_L^{\bullet+}$ interacting with a neutral BChl P_M. The unpaired spin on P_L induces the transition on P_M, causing its intensity to be strongly dependent on the electronic coupling between the two moieties. Therefore, the reduction of the oscillator strength of the 1250-nm band for $P_{850}^{\bullet+}$ by a factor of about 3 could also be indicative for a decoupling of the two dimer halves in this state, i.e., a decrease of β_D. On the basis of the assumption that the decoupling of the dimer is achieved by increasing the distance between the two macrocycle planes, the model calculations of Reimers and Hush (1995a,b) allow us to assess the magnitude of this distance change to be approximately 0.3 Å. It is conceivable that such slight changes can be induced by conformational reorientation of the protein environment without a major distortion of the geometries of the macrocycles as discussed above. It would be of great interest to investigate the effects of zwitterionic detergents on transitions of $P^{\bullet+}$ in the mid-IR region in order to further evaluate changes of $\Delta\alpha$ and/or β_D [cf. Breton et al. (1992)]. As is also shown by semiempirical calculations (Warshel & Parson, 1987; Thompson & Zerner, 1991), the optical properties of the neutral states P_{850} and P_{866} are fully consistent with the interpretation that the distance between the dimer halves is changed.

In principle it is possible to change the EPR linewidths continously, in a way similar to the position of the long-wavelength absorption maximum, by changing the detergent/RC ratio. Such a behavior could imply that a manifold of protein conformations exists and that one can adjust every value of ρ_L/ρ_M between 2 and 5.5 by addition of zwitterionic or charged detergents. Correspondingly, the hfcs should shift continously. However, the spectra of Figures 2 and 4 clearly show that this is not the case. In contrast one can identify two distinct sets of hfcs that are sufficient to fully explain the spectra. The two sets of hfcs are identical to the two extreme cases discussed above. Thus, there exist *only two* different conformations of $P^{\bullet+}$ that can clearly be distinguished by their hfcs. This does not exclude the existence of subconformers in each of the two states. However, these subconformers cannot differ significantly in the electronic structure of $P^{\bullet+}$, but rather cause inhomogenous broadening of the observed resonance lines. For example, the signals of the β-protons at positions 3, 4, 7, and 8 are significantly broadened as compared with those of the methyl protons. This can be traced back to the conformational flexibility of the saturated rings II and IV.

The coexistence of two distinct states in the range $\lambda_2 < \lambda_{max} < \lambda_1$ implies that the optical bandshift must be interpreted as a transition between two states with displaced but fixed absorption maxima. A change of the detergent/RC ratio influences the relative amplitude of the bands, thus causing an apparent shift of λ_{max}. Since the widths of the bands are larger than their displacement, no isosbestic point or shoulder can be seen.

The main property that determines the ability of the detergent to induce P_{850} is the polarity of the head group. This can be inferred from a direct comparison of detergents with different head groups, but the same hydrophobic part, e.g., Brij 36 T, LDAO, and SB12 [for a recent discussion of detergent properties, see Laughlin (1994)]. Our experiments show that a lower detergent/RC ratio is required to stabilize P_{850} in SB12 than in LDAO. Similarly, nonionic detergents are not even able to shift λ_{max} at all, while ionic detergents like CTAB or sodium dodecyl sulfate (Agalidis, 1987) have a strong impact on λ_{max}. We therefore think that the electric field of the head group is the main cause of the conformational change. This field could lead to a reorientation of polar side chains in the outer parts of the RC protein and/or a readjustment of α-helices, which in turn influences the protein environment of P. The behavior of the bile salts can be understood by considering their different molecular structure and aggregation behavior (Helenius & Simons, 1975). It is conceivable that in these cases only a small number of molecules is bound to the RC, which is not sufficient to induce considerable amounts of P_{850}. Another important factor that influences the relative stability of the two conformations could be steric constraints imposed on the protein by the detergent belt or by aggregation due to removal of the detergent. For example, detergent-free RCs prepared according to Gast et al. (1996) also exhibit the typical spectroscopic features of $P_{850}^{\bullet+}$ (Rautter, 1995).

In the present study, we used simple dialysis for detergent exchange. Gast et al. (1994) found that upon dialysis of RCs from *R. viridis* in the absence of another detergent <20% of the LDAO remains bound to the protein. In contrast, a Bio-Beads treatment removes 95% of the detergent. We performed the dialysis in the presence of a high excess of another detergent, which should effectively compete with the LDAO for binding to the RC. Although we cannot exclude that traces of LDAO are present in our samples, we found no differences between the spectroscopic properties of the RCs, when performing the detergent exchange according to the two different methods. The main

reason for applying simple dialysis was to maintain a high yield of intact RCs.

In Figures 2 and 4, the detergent concentration before ultrafiltration (dialysis buffer) is given instead of the detergent/RC ratio of the actual concentrated sample. By concentrating the RCs using Centricon 30 tubes, a certain percentage of the detergent may pass the membrane. We have not analyzed quantitatively the dependence of the mole fraction of $P_{850}^{\bullet+}$ on the SB12/RC ratio. This was done in our earlier optical titration experiments (Müh et al., 1996). Nevertheless, we kept the RC concentration and all other conditions constant. The corresponding TRIPLE spectra show a clear correlation between the SB12 concentration in the original dialysis buffer and the amount of $P_{850}^{\bullet+}$ in the actual sample. This indicates a quite uniform change of the SB12/RC ratio of all samples upon ultrafiltration. Therefore, we can qualitatively discuss the observed effects in analogy to the optical titration experiments.

The equilibrium between the two conformations of the RCs is characterized by a standard Gibbs free energy difference

$$\Delta G^\circ = -RT \ln K \tag{2}$$

where $K = X/(1 - X)$ is the equilibrium constant for the transition from P_{866} to P_{850}. For the sulfobetaines the experiments show that K depends on the detergent/RC ratio ζ and the temperature T. The data imply that a certain value ζ_0 of the detergent/RC ratio exists, for which the free energy difference vanishes. From eq 2 and the equation for the titration curves published earlier (Müh et al., 1996), i.e.,

$$\zeta = \zeta_0 + \frac{RT}{\kappa} \ln K \tag{3}$$

one obtains the simple relationship

$$-\Delta G^\circ = \kappa(\zeta - \zeta_0) = \kappa \Delta\zeta \tag{4}$$

where κ has the dimension of energy per mole. An increase of ζ, which causes a more positive $\Delta\zeta$, yields a more negative ΔG°. According to the definition of K, this in turn leads to a stronger preference for P_{850}, as is confirmed by the experiment. The depth of the free energy gap that is caused by a certain value of $\Delta\zeta$ is determined by the parameter κ. Both κ and ζ_0 depend on the detergent properties. In particular, κ should be a function of the polarity of the detergent head group. According to this interpretation the number of detergent molecules that are bound to the RC determines ΔG° (at least in the case of the sulfobetaines). Consequently, the change of K between 278 and 298 K can be interpreted as a slight decrease of the number of molecules in the detergent belt with increasing temperature. The corresponding increase of ΔG° in favor of P_{866} could then be traced back to a temperature dependence of ζ_0 and/or κ in eq 4. Note that this simple model only applies when the phase behavior of the detergent does not change. In the case of SB12, even extremely concentrated (50%) solutions in water form a micellar liquid phase in the investigated temperature range (Nilsson et al., 1984).

While freezing of RC solutions containing nonionic detergents or sulfobetaines appears to have no effect on the conformation of $P^{\bullet+}$, a complete switch from $P_{866}^{\bullet+}$ to $P_{850}^{\bullet+}$ occurs upon freezing of LDAO containing solutions without cryprotectants. This indicates either a change of the detergent−RC interaction or a dramatic increase of the local LDAO concentration near the RC due to phase separation. In both cases, trioles like glycerol can in principle prevent the formation of $P_{850}^{\bullet+}$ by either entering the detergent belt and disturbing the detergent−RC interaction or changing the phase behavior of the system. For example, it is known that heptanetriol reduces the number of LDAO molecules bound to the RC (Gast et al., 1994, 1996). Tiede et al. (1995) observed that aggregation of LDAO-solubilized RCs due to phase separation is not recovered immediately upon thawing, but can take several hours. This may be related to our observation of $\lambda_{max} < 866$ nm under these conditions.

It is noteworthy that the existence of two states of the primary donor is a conserved property of RCs from several species of nonsulfur purple bacteria. Rautter et al. (1994) found $P_{850}^{\bullet+}$ in RCs from *R. capsulatus* and *R. centenum*. The possibility to interconvert the RCs between the two forms using detergents was demonstrated for *R. capsulatus* by Wang et al. (1994) and Rautter et al. (1994) and for *R. sphaeroides* in this work. A detergent-induced blueshift of λ_{max} can also be observed in the Bchl *b*-containing RCs from *R. viridis* (F. Müh, unpublished). Although the exact experimental conditions for the occurrence of the second state differ from species to species, the spectroscopic characteristics of the two states are always the same. This suggests that the change of the electronic and spatial structure of the primary donor is not simply an artifact of the solubilization, but rather could play a role in RC function.

Recently, we observed that the rate of charge recombination from $P^{\bullet+}Q_A^{\bullet-}$ in RCs from *R. sphaeroides* can be reduced by up to 40% by addition of SB12 (F. Müh, E. Schlodder, W. Lubitz, unpublished results). The second state of P could therefore possibly be involved in the stabilization of charge-separated states. Parot et al. (1987) found indications for the presence of two RC conformations at 10 K in chromatophores, which could be related to the formation of P_{850}. It is also possible that a conformational change like the one observed in this study plays a role in the fast unidirectional foward electron transfer and contributes to the complexity of the observed kinetics [see, e.g., Hamm & Zinth (1995) and Hartwich et al. (1996)]. Experiments are in progress in our laboratory to answer these open questions.

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