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Characterization of Pheophytin Ground States in *Rhodobacter sphaeroides* R26 Photosynthetic Reaction Centers from Multispin Pheophytin Enrichment and 2-D ^{13}C MAS NMR Dipolar Correlation Spectroscopy†

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ABSTRACT: The electronic ground states of pheophytin cofactors potentially involved in symmetry breaking between the A and B branch for electron transport in the bacterial photosynthetic reaction center have been investigated through a characterization of the electron densities at individual atomic positions of pheophytin *a* from ^{13}C chemical shift data. A new experimental approach involving multispin ^{13}C labeling and 2-D NMR is presented. Bacterial photosynthetic reaction centers of *Rhodobacter sphaeroides* R26 were reconstituted with uniformly ^{13}C biosynthetically labeled (plant) Pheo *a* in the two pheophytin binding sites. From the multispin labeled samples 1-D and 2-D solid-state ^{13}C magic angle spinning NMR spectra could be obtained and used to characterize the pheophytin *a* ground state in the *Rb. sphaeroides* R26 RCs, i.e., without a necessity for time-consuming selective labeling strategies involving organic synthesis. From the 2-D solid state $^{13}C-^{13}C$ correlation spectra collected with spinning speeds of 8 and 10 kHz, with mixing times of 1 and 0.8 ms, many ^{13}C resonances of the [U-^{13}C]Pheo *a* molecules reconstituted in the RCs could be assigned in a single set of experiments. Parts of the pheophytins interacting with the protein, at the level of ^{13}C shifts modified by binding, could be identified. Small reconstitution shifts are detected for the 17^2 side chain of ring IV. In contrast, there is no evidence for electrostatic differences between the two Pheo *a*, for instance, due to a possibly strong selective electrostatic interaction with Glu L104 on the active branch. The protonation states appear the same, and the NMR suggests a strong overall similarity between the ground states of the two Pheo *a*, which is of interest in view of the asymmetry of the electron transfer.

Understanding photosynthesis at the atomic level is a challenge in structural biology today. It is of importance to characterize the electronic structure in the ground state and the relation to the spatial structure and structure−function properties of cofactors in photosynthetic proteins, which will be of help to reveal their roles in the molecular mechanisms of photochemical energy conversion (Deisenhofer *et al*., 1995; Scheer *et al*., 1992; Kühlbrandt, 1994; de Groot, 1996; Gerwert, 1995). The reaction center (RC)[1] of the photosynthetic bacterium *Rhodobacter (Rb.) sphaeroides* R26 is a transmembrane protein complex that consists of three polypeptide chains (L, M, and H) supporting the cofactors: four bacteriochlorophylls, two bacteriopheophytins (Φ_A and Φ_B), two ubiquinones, and one Fe^{2+} ion (Figure 1). Following the pioneering work of Deisenhofer and Michel (1986), the complex has been crystallized and the structure of the complex is now known in great detail, with up to 2.65 Å resolution (Rees *et al*., 1989; Chang *et al*., 1991; Ermler *et al*., 1994; Arnoux *et al*., 1995). The cofactors are located in the hydrophobic transmembrane segment formed by the L and M subunits and form two nearly symmetric branches, denoted by A and B. The photochemistry involves the transfer of electrons from a bacteriochlorophyll dimer (P) at the periplasmic side of the RC over the A branch to the ubiquinones, first to Q_A and subsequently to Q_B. The cofactors are supported by helices of the L and M subunits in the transmembrane segment of the complex that is strongly nonpolar and rigid (Rees *et al*., 1989; Chang *et al*., 1991; Ermler *et al*., 1994; Arnoux *et al*. 1995).

Intensive study of the RC has provided detailed insight into the functional role of the prosthetic groups, their connection with electron pathways, and the kinetics of various electron transfer steps. The vast majority of experi-

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⊥ EMBL.

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[1] Abbreviations: B, bacteriochlorophyll monomer; BPheo *a*, bacteriopheophytin *a*; CP, cross-polarization; DEAE, diethylaminoethyl; EDTA, ethylenedinitrilotetraacetic acid; ENDOR, electron nuclear double resonance; LDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide; MAS NMR, magic angle spinning nuclear magnetic resonance; P, bacteriochlorophyll dimer; PAGE, polyacrylamide gel electrophoresis; Pheo *a*, pheophytin *a*; Φ, bacteriopheophytin monomer; Q, quinone; *Rb. Rhodobacter*; RC, reaction center; RFDR, radio frequency-driven dipolar recoupling; RP HPLC; reverse phase high-performance liquid chromatography; THF, tetrahydrofurane; Tris, Tris-hydroxymethylaminomethane; U-^{13}C, uniformly ^{13}C enriched.

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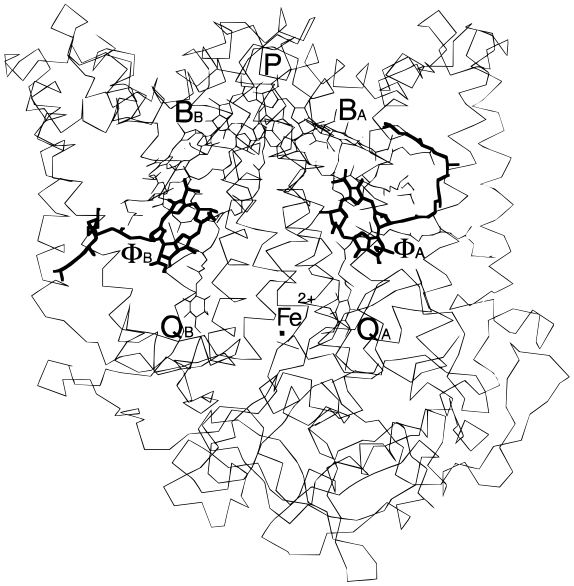

FIGURE 1: Arrangement of the cofactors in *Rb. sphaeroides* R26 RC (Ermler *et al*., 1994). For this study, the two BPheo *a* (Φ_A and Φ_B) were exchanged by [U-^{13}C]Pheo *a*.

mental effort to date has been devoted to reaction centers from *Rb. sphaeroides* [see, e.g., Kirmaier and Holten (1987)]. The two pheophytins in the *Rb. sphaeroides* RC are chemically identical, but only one, Φ_A, is known to function as an intermediary electron acceptor during the electron transfer proceeding from the donor P to the primary electron acceptor quinone Q_A, while the other pheophytin, Φ_B, appears not to be actively involved in electron transfer (Allen *et al*., 1987; Scheer *et al*., 1992). It is thus of interest to study if and how the ground states of the pheophytins are influenced by the surrounding protein, since this could in principle be instrumental in provoking electron transfer via the L but not via the M branch.

In order to understand the properties of *Rb. sphaeroides* RC, the relationship between the structure of the RC and its function at a molecular level has been investigated with mutagenesis or modification of RCs followed by spectroscopic analysis [see, e.g., Kirmaier and Holten (1987, 1993), Gray *et al*. (1992), and Shochat *et al*. (1995)]. Pigment modification is a relatively recent tool to study structure−function relationships of purple bacterial RCs. The replacement of BChl by BPheo is possible by site-directed mutagenesis of the apoproteins (Coleman & Youvan, 1990; Schenck *et al*., 1990; Woodbury *et al*., 1990). More extensively modified pigments can be introduced by an exchange procedure. For instance, chemically modified tetrapyrroles were incorporated into the RC (Scheer & Struck, 1993; Scheer & Hartwich, 1996; Meyer & Scheer, 1995). Recently, it has been shown by Meyer and Scheer (1995) that RCs containing BPheo *a*, [3-vinyl]BPheo *a*, Pheo *a* or [3-acetyl]Pheo *a* are capable of reversible light-induced electron transfer, although with decreased efficiency. A detailed kinetic analysis of RCs with Pheo *a* in both sites indicates that the relative order of redox potentials measured in solution is maintained in the Φ_A binding site (Shkuropatov & Shuvalov, 1993; Schmidt *et al*., 1994).

The present investigation aims at providing a next step into the study of the protein-pheophytin interactions in *Rb. sphaeroides* RCs through a partial characterization of the electronic ground state structure with multispin isotope labeling of Pheo *a* followed by 1- and 2-D ^{13}C CP/MAS NMR of the [U-^{13}C]Pheo *a* reconstituted into *Rb. sphaeroides* R26 RCs. Previously, it was shown that NMR is a good technique to probe electronic ground states of cofactors. For the RC complex with a molecular mass exceeding 100 kDa, atomic selectivity was obtained using high-resolution CP/MAS NMR to study specifically enriched samples (de Groot *et al*., 1990, 1992, 1995).

Recently, it was shown that uniformly enriched samples of Chl *a* and BChl *c* yield highly resolved 2-D dipolar correlation spectra that provide a complete spectral assignment and therefore detailed insight into the electronic ground state structure with atomic selectivity (Boender *et al*., 1995; Balaban *et al*., 1995). We here introduce a novel concept of multispin labeling and assignment of ^{13}C CP/MAS resonances of ^{13}C clusters counting up to ~1 kDa in a large protein complex. Both BPheo in the RCs can be replaced by [U-^{13}C]Pheo *a*, since the [U-^{13}C]Pheo *a* can be obtained in a sufficient amount (10−50 mg) for RC reconstitution studies from the plant chlorophyll that becomes available when growing uniformly enriched algae cultures for the preparation of expression media for solution NMR studies. Collecting 1-D and 2-D correlation data of the RC reconstituted with the U-^{13}C-labeled Pheo *a* yields the assignment of many ^{13}C resonances without the necessity for time-consuming organic synthesis strategies for selective isotope enrichment. It allows the study of the electronic structure, polarization, and protonation/hydrogen bonding state of the Pheo *a* in the RC. This provides another step toward the detailed characterization of pheophytin binding in the RC, in relation to the function. In particular, our data suggest a great deal of similarity between the two pheophytins in terms of local atomic charge densities for ring carbons.

MATERIALS AND METHODS

To obtain uniformly enriched Pheo *a*, *Chenopodium rubrum* was grown on a ^{13}C-labeled source medium (Prytulla, 1994). Eight identifiable pigments, β-carotene, lutein, chlorophyll *a* and *b*, pheophytin *a* and *b*, violaxanthin, and neoxanthin could be isolated using silicagel column elution with pure hexane, followed by elution with hexane/acetone, 6/4. The purity of the pigments was controlled by absorption spectroscopy, analytical RP HPLC, and NMR spectroscopy. An additional amount of [U-^{13}C]Pheo *a* was obtained by bubbling a stream of N_2 containing gaseous HCl into the ether solution of Chl *a* as described in Watanabe *et al*. (1984). About 10 mg of labeled Pheo *a* was used for exchange with BPheo *a* in *Rb. sphaeroides* RCs.

The RCs from *Rb. sphaeroides* R26 were isolated by treatment of chromatophores with LDAO, followed by purification with DEAE-Sephacel chromatography (Feher & Okamura, 1978). Replacement of the BPheo by labeled Pheo *a* has been done using a slightly modified method described earlier (Scheer *et al*., 1992; Shkuropatov *et al*., 1994). Briefly, 22 batches of 10 mL each containing about 14 μM RC, 50 μM labeled Pheo *a*, 0.1% LDAO, 1 mM EDTA, 10 mM Tris, pH 8, and 10% acetone were incubated for 90 min at 42 ± 0.5 °C. After incubation, the batches were combined and put on ice. Denatured protein was removed by centrifugation for 10 min at 6000*g*. The RCs were loaded on a DEAE-sephacel column and washed with 60 mM NaCl, 0.1% LDAO, 10 mM Tris, pH 8, 1 mM EDTA. Subse-

quently, they were removed from the column with 400 mM NaCl. After dialysis overnight *vs* 0.025% LDAO, 10 mM Tris, pH 8, and 1 mM EDTA, the RCs were again loaded on a DEAE-Sephacel column and removed from the column using a salt gradient 0−500 mM NaCl, 0.025% LDAO, 10 mM Tris pH 8, 1 mM EDTA. After a second dialysis, the RCs were concentrated using a 100 kDa filter and a 4 mm MAS rotor was loaded with about 40 mg of RCs. The RCs had a 280/800 absorption ratio of 1.4, and SDS−PAGE demonstrated that all three protein subunits (L, M, and H) were still present. The final yield of ^{13}C Pheo *a* reconstituted RCs was about 10%. Detergent free RCs were obtained according to the method described in van Liemt *et al*. (1995).

For the CP/MAS NMR experiments, a commercial MSL400 spectrometer (Bruker) equipped with a double resonance 4 mm CP/MAS probe (Bruker, Karlsruhe, Germany) was used. The spinning rate about the magic angle was stabilized with a home-built spinning speed controller (de Groot *et al*., 1988). 1-D CP/MAS spectra were collected with a contact time of 2 ms at a ^{13}C−^{1}H Hartmann−Hahn matching condition (Pines *et al*., 1973). Spinning speeds of 7000, 8000, 10 000, and 11 000 Hz were used to assign side bands. For the 2-D ^{13}C dipolar correlation spectroscopy experiments, variable amplitude cross-polarization (VACP) with a contact time of 2 ms was used (Peersen *et al*., 1993). During the VACP contact time, the ^{1}H power level was varied between 35 and 65% of the decoupling power. Prior to the 2-D correlation experiment, the VACP was optimized by adjusting the ^{13}C power for maximum signal intensity from a sample of [1-^{13}C]glycine. Absorption mode dipolar correlation spectra were measured using procedures described in Boender *et al*. (1995). This method is based on the RFDR pulse sequence developed by Bennett *et al*. (1992). The NMR spectra of the RCs were recorded at −50 °C in order to immobilize the sample by freezing. The cycle time between scans was typically 1 s, and data were acquired with continuous-wave ^{1}H heteronuclear dipolar decoupling with a nutation frequency of ∼60 kHz.

An external calibration against the 1-^{13}C signal of glycine at 176.04 ppm was used. 1-D and 2-D ^{1}H and ^{13}C NMR spectra of [U-^{13}C]Pheo *a* in acetone-d_6 were collected with a Bruker 600 MHz DMX spectrometer. Optical (difference) spectra at cryogenic temperature were performed with a single-beam spectrophotometer, as in Smit *et al*. (1987). Absorption differences were induced by flash excitation at 890 nm.

RESULTS AND DISCUSSION

Pheo *a* is the electron acceptor in the photosystem II D1/D2 complex, which is believed to resemble the bacterial RC (Michel & Deisenhofer, 1988). It differs from BPheo *a* in the hydrogenation state of ring II and in the substituent at C-3 (Figure 2). The percentage of BPheo replacement in the RC of *Rb. sphaeroides* and the ratio of active/nonactive branch replacement can be determined by recording the low-temperature absorption and the $P^+Q_A^-$ absorption−difference spectrum (Franken *et al*., 1997).

The absorption spectra at 10 K of native and ^{13}C Pheo *a* reconstituted RCs are depicted in Figure 3A. Around 535 and 545 nm, the two Q_x absorption bands of Φ_A and Φ_B are well separated in the native RCs at this temperature. In the

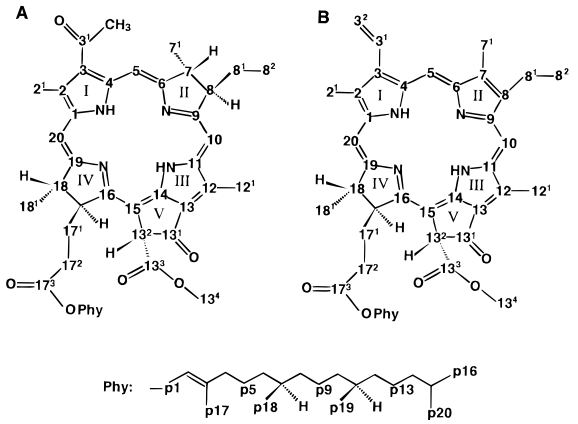

FIGURE 2: Chemical structures of BPheo *a* (A) and Pheo *a* (B) with the IUPAC numbering scheme.

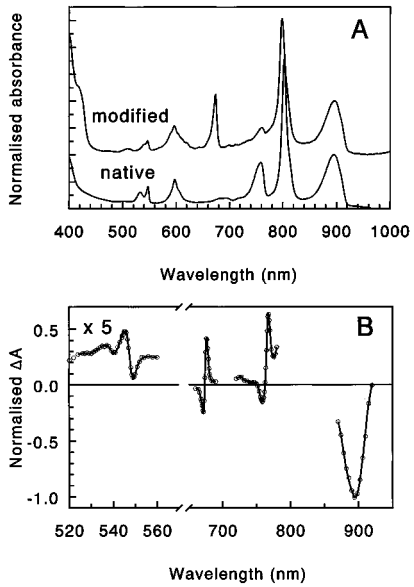

FIGURE 3: (A) Absorption spectra of native and [U-^{13}C]Pheo *a*-reconstituted RCs from *Rb. sphaeroides* R26 at 10 K; (B) absorption difference spectrum at 10 K of $P^+Q_A^-$ state, recorded 1 ms after laser excitation at 890 nm.

modified RCs, the 535 nm band is virtually absent while the 545 nm band is reduced. This shows that almost all Φ_B has been replaced. The relative amount of Φ_A replacement is more difficult to estimate from the absorption peak at 545 nm, since the reconstituted Pheo *a* also absorbs at the same wavelength (Shkuropatov *et al*., 1994). From the ratio of the peaks at 676 nm (Pheo *a*) and 890 nm (P), we obtain that 88 ± 5% of the total amount of BPheo has been replaced by labeled Pheo *a*. This may represent a slight overestimation since free and nonspecifically bound Pheo *a* also absorbs at 676 nm. Finally, the absorption band at 760 nm indicates that the amount of nonreplaced BPheo is less than 20%. Thus, the overestimation based on the absorption at 676 nm is minor, and less than 10% of free or nonspecifically bound [U-^{13}C]Pheo *a* is present in this sample. Figure 3B shows the absorption changes in modified RCs due to the formation of $P^+Q_A^-$ charge separation upon illumination. The negative peak at 549 nm and the band shift at 675 nm are the best indications for the percentage of BPheo replacement by Pheo *a*. The peak at 549 nm only appears in RCs in which both BPheo have been replaced (Franken *et al*., 1997). By comparing the changes at 549 nm relative to the bleaching at 895 nm found in this sample, it is concluded that still 12

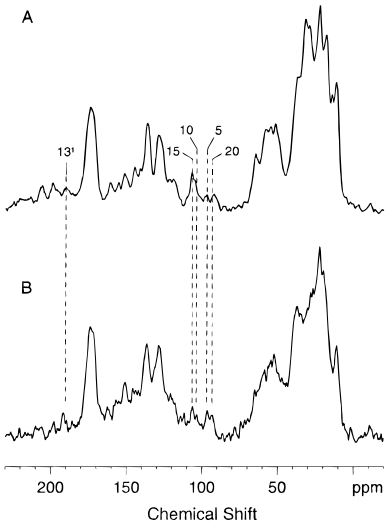

FIGURE 4: Proton-decoupled ^{13}C CP/MAS spectra of *Rb. sphaeroides* R26 RCs reconstituted with U-^{13}C-labeled plant Pheo *a* recorded at a temperature of 230 K and a spinning speed of 7000 Hz (A). A spectrum at 11 000 Hz (B) was collected at ambient temperature from a detergent-free sample. The dashed lines indicate the positions of the methine and 13^1 responses in the spectra, according to the assignment in Table 1.

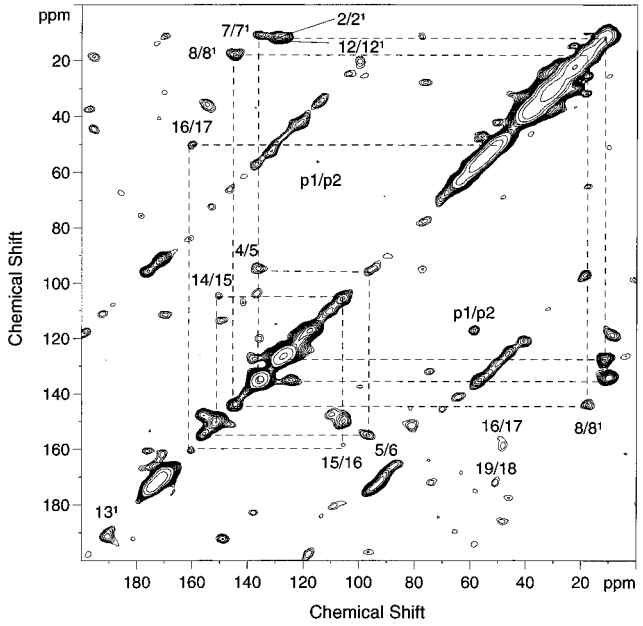

FIGURE 5: Contour plot of a MAS 2-D ^{13}C NMR dipolar correlation spectrum of *Rb. sphaeroides* R26 RCs reconstituted with U-^{13}C-labeled plant Pheo *a* recorded at a temperature of 230 K and spinning speed of 8000 ± 3 Hz and with a mixing time τ_m=1 ms. The dashed lines indicate extended nearest-neighbor correlation networks.

± 10% of native BPheo *a* is present (Franken *et al*., 1997). From the relative amplitude of the band shift at 675 nm compared to the bleaching at 895 nm, we conclude that 75 ± 10% of BPheo *a* has been replaced. Thus, from Figure 3, panels A and B, we conclude that in this sample all Φ_B and 65 ± 8% of the Φ_A have been replaced by [U-^{13}C]Pheo *a*, of which ca. 90% is bound to the RCs. The error margin represents the statistical average of the two independent measurements.

The reconstitution procedure provides an opportunity to perform a comparative study of both the active and the inactive pheophytin protein binding site with exactly the same multispin labeled NMR probe, the [U-^{13}C]Pheo *a*. Investigation of protein−Pheo *a* interactions is made by collecting 1-D CP/MAS and 2-D CP/MAS dipolar correlation data of both multispin clusters simultaneously, at low temperature and with high spinning speeds. 1-D CP/MAS spectra at natural ^{13}C abundance allow the assignment of only a few signals. For instance, the 5, 10, 15, 20, and 13^1 peaks can be identified (Figure 4). A more comprehensive ^{13}C assignment necessary to study the electronic structure, polarization, and protonation/hydrogen bonding states can be obtained from the analysis of the connectivities in 2-D correlation data. Assignment of resonances through dipolar correlation spectroscopy has been performed previously on moderately sized uniformly labeled systems (Boender *et al*., 1995; Balaban *et al*., 1995). In the present study, we introduce clusters of labels, in the form of two uniformly labeled Pheo *a*, in a much larger protein complex. The assignment of ^{13}C resonances using multispin labeled systems offers considerable advantages compared to single isotope studies, since there is no need for using selectively labeled cofactors that can only be obtained through elaborate expert organic synthesis procedures. In addition, when it is possible to assign the response of several connected carbons of a cofactor in a large protein complex, a more global picture with respect to protein−cofactor interactions is obtained, in terms of shifts that occur relative to the response of the labeled molecule in solution. Although individual shifts may then be small and difficult to interpret, the systematic occurrence of small shifts in a certain region of the molecule can reveal parts of the cofactor interacting with the protein surrounding.

Figure 5 shows an example of a 2-D dipolar correlation spectrum of the reconstituted RCs. In this plot, the contour levels are chosen well above the background noise. MAS side band diagonals at $\omega_r/2\pi = 8000 \pm 5$ Hz are clearly visible as well as some strong 2-D cross peaks revealing transfer of coherence. Several of the cross peaks and diagonal peaks are connected by lines illustrating how they contribute to a correlation network revealing the molecular framework, similar to the assignment procedures in solution NMR (Boender *et al*., 1995). The high spinning speed used for the experiment in Figure 4 minimizes the occurrence of rotational resonance effects and of overlap between the correlated peaks and sideband lines.

In Figure 5 only the strongest cross peaks are plotted. As was demonstrated previously by going to lower intensity levels and using automated peak identification procedures that are routinely available nowadays from solution NMR analysis packages, information about many cross peaks can be extracted from such a 2-D MAS spectrum (Boender *et al*., 1995). For a short mixing time, ∼1 ms, the correlations are predominantly associated with nearest-neighbor carbon−carbon connectivities. Assignment of Pheo *a* resonances was based on a detailed analysis of two spectra collected with spinning speeds of 8000 Hz and 10 000 Hz. Only nearest-neighbor correlations symmetrically present on both sides of the diagonal or present in both 2-D data sets were taken from the noisy background and used for the assignments. After the elimination of noise artifacts and side band correlations, the assignment procedure led to the identification of several extended nearest-neighbor ^{13}C correlation networks: C14 (151)−C15 (107)−C16 (161)−C17 (52.0)−$C17_1$ (32); C13 (133)−$C13^1$ (190)−$C13^2$ (64)−$C13^3$ (171); C4 (137.0)−C5 (97)−C6 (156)−C7 (136)−$C7^1$ (11); C3

(136)−C3^1 (129)−C3^2 (121); C9 (150)−C10 (105)−C11 (138)−C12 (128)−C12^1 (12). In addition, in the phytyl chain of Pheo *a* there are the correlations C17^2 (29)−C17^3 (173)−p1 (59)−p2 (117)−p3 (144)−p4 (41) and there is also a cross peak between p3 and p17 (17). Finally, the 13^1 response, with $\delta_i = 190$ ppm, is marked.

Some of these correlation networks involving the strongest cross peaks are depicted in Figure 5 with the dashed lines. Note that the shifts of carbons 3 and 4 appear to be identical within the accuracy of the experiment. It appears that in both spectra, 2/2^1 and 12/12^1 are strongly overlapping. Some other correlations are less readily identified. For instance, 10/9 appears to overlap with 14/15. From the 1-D spectra, at least part of the spectral intensity appears at 104.7 ppm. However, the 2-D spectra are somewhat ambiguous and leave room for an additional assignment to a response at 107.3 ppm. The difference does not affect the global interpretation of the results. From the other 2-D spectrum at 10 000 Hz spinning speed also the chemical shifts for 17^2/17^3 and p3/p17 could be assigned (data not shown). In the ring IV of the Pheo *a* molecules a correlation between C18 (50) and C19 (173) could be identified, but it is possible that the same cross peak corresponds to a two-bond C13^3/C13^4 cross peak. Finally, there is a correlation between the C8 (145) and C8^1 (19). A mandatory condition for narrow intense cross peaks is a rigid well-ordered solid sample. Thus, it is not surprising that the ends of the phytyl chains were not detected, considering that they are likely more disordered in the frozen NMR sample.

In Table 1 the solid state shifts for the Pheo *a* in the RC are compared with data for the molecule in acetone-d_6 and THF-d_8 (Lötjönen & Hynninen, 1983). The use of two different nonprotic solvents for the solution studies allows a quantification of pure solvent effects in terms of chemical shifts. For the ^{13}C responses listed in Table 1, the shift variations due to effects associated with the two different solvents are less than 1 ppm, comparable to the estimated accuracies for the solid state shifts, which are ∼1 ppm.

The most recent X-ray data indicate undistorted rings and similar protein−cofactor interactions for the two BPheos (Ermler *et al*., 1994). In addition, Meyer *et al*. (1996) have shown that the Pheo *a* in the reconstituted RC are oriented similar to the native ones and that they do not affect significantly the linear dichroism. The solid state NMR results are in line with the results from these studies, since most shifts are very close to the values measured in solution, while pronounced distortions of the molecule, for instance differences in ring puckering, would imply considerable reconstitution shifts and possibly differences between the two sites. Also protein−cofactor interactions affecting the protonation state of the nitrogens would give rise to substantial reconstitution shifts at the 1, 4, 11, and 14 positions and are therefore unlikely (Lötjönen & Hynninen, 1983). When the solid state chemical shifts are compared with the shifts for the Pheo *a* in solution, small differences appear for carbons 17^2, p1, and p2. The data thus provide evidence for weak protein−Pheo *a* interactions affecting the 17 side chain and the first part of the phytyl moiety, which could be distorted by the protein.

The two BPheos in the native RC are slightly different spectrally in the Q_x region, and only the BPheo absorbing at longer wavelength, associated with the L subunit (Φ_A), is active in electron transfer [see, e.g., Clayton and Yamamoto (1976) and Bylina *et al*. (1988)]. The most important structural difference between the sites of Φ_A and Φ_B is the presence of the L104 glutamic acid residue near ring V of the photoactive Φ_A. This glutamic acid residue is conserved between various bacterial L subunits and is also present in the D1 protein in photosystem II, which is thought to be the equivalent of the L subunit in the bacterial reaction center (Michel *et al*., 1986; Hearst, 1986). Resonance Raman, infrared, and ENDOR data all indicate that Φ_A interacts with the Glu L104 (Bocian *et al*., 1987; Nabedryk *et al*., 1988; Feher *et al*., 1988). On the other hand, functional studies of site-specific mutants have clearly shown that other amino acids in position L104 only moderately influence the dynamics and unidirectionality of the electron transfer (Bylina *et al*., 1988). Finally, the glutamic acid residue is in a hydrophobic environment, and does not have a countercharge in its neighborhood. It is therefore most likely protonated and neutral (Michel *et al*., 1986). This is in line with the NMR data. There is at the present state of the technology no evidence for a selective polarization of ring V of one of the pheophytins. In particular, the 13^1 response

Table 1. Partial Assignment of ^{13}C NMR Signals of [U-^{13}C]Pheo *a* in *Rb. sphaeroides* RCs (δ_c)[a]

no.	δ_{THF} (ppm)	$\delta_{acetone}$ (ppm)	δ_c (ppm)
7^1	10.8	10.9	11
12^1	11.7	11.2	12
2^1	11.9	11.9	12
p17	16.2	16.2	17
8^2	17.4	17.7	17
8^1	19.5	19.8	19
18^1	23.3	23.5	22
p5	25.8	25.3	26
17^1	30.2	30.3	32
17^2	31.7	31.7	29
p4	40.5	40.1	41
18	50.8	50.8	50
17	52.1	52.1	52
p1	61.4	61.2	59
13^2	66.4	65.8	[64]
20	93.9	94.6	93
5	97.5	97.8	97
10	104.5	105.3	105
15	106.8	107.1	107
p2	119.5	119.1	117
3^2	122.1	123.1	121
12	128.9	128.9	128
3^1	129.7	129.8	129
13	130.1	130.5	[133]
2	132.1	132.7	131
7	[136.0]	136.8	136
3	[136.4]	136.5	136
4	[136.5]	136.7	137
11	138.5	138.5	138
1	142.2	142.2	142
p3	142.2	142.3	144
8	145.2	146.0	145
14	149.9	149.9	151
9	151.4	151.3	150
6	155.6	156.1	156
16	162.1	162.2	161
13^3	169.9	170.3	171
19	172.8	173.6	173
17^3	172.9	172.8	173
13^1	189.2	189.8	190

[a] Solution shifts in THF-d_8 (δ_{THF}) were taken from Lötjönen and Hynninen (1983), $\delta_{acetone}$ (ppm) is the solution shift in acetone-d_6 (this work). The accuracy of the solid state shifts is ∼1 ppm. The numbering is according to the scheme in Figure 2. Assignments in square brackets are tentative.

at 190 ppm, which is well resolved in both the 1-D and 2-D datasets, is not split. It parallels the ENDOR investigations of reduced BPheo in the bacterial and PSII RCs, which indicated that the effects of a hydrogen-bonded Glu L104 on the electronic structure of the BPheo in the native system are small (Feher *et al*., 1988; Lubitz *et al*., 1989).

There has been considerable speculation as to whether the spectroscopic red shift of the Q_x band of Φ_A can be attributed to a specific interaction with the Glu L104 [for examples, see Michel *et al*. (1986) and Bocian *et al*. (1987)]. For instance, Glu L104 was changed to leucine, glutamine, and lysine in RCs of *Rb. capsulatus* and low-temperature absorption spectra of these genetically modified RCs indicate that a Glu L104 residue is probably necessary for the spectroscopic red shift of the photoactive BPheo. According to the X-ray structure, the Glu L104 is located within hydrogen-bonding distance of the keto carbonyl group of the ring V of Φ_A. In addition, the ENDOR investigations of the reduced BPheo *a* in *Rb. sphaeroides* R26 RCs have provided convincing evidence for a hydrogen bond between the Φ_A keto functionality and the conserved Glu L104 (Feher *et al*., 1988). It has been proposed that this hydrogen bond is a prerequisite for the red-shifted absorption of Φ_A compared with Φ_B in the native system (Bylina *et al*., 1988). It is thus remarkable that, for the Pheo *a* reconstituted RC, both Φ_A and Φ_B exhibit a band around 545 nm, since this suggests that a hydrogen bond with Glu L104 may not be the only structural motif essential for the red shift of Φ_A, or that there is no H bond in neutral Φ_A. We have performed semiempirical quantum chemical calculations for Pheo *a in vacuo* and solvated at the keto group with a hydrogen-bonded water or acetic acid molecule. The charge effect from the hydrogen bond would translate into a small down field shift of ~4 ppm, while the charge effect from a change in hydrogen-bonding partner would be ~1 ppm. Such shift effects are small, on the order of the line width, and are difficult to assess in RC with both Pheo replaced. However, selective replacement of only one Pheo may be possible and will be investigated in future.

For the pure Pheo *a* in solution, the effect of the solvent on the chemical shifts can be measured more accurately than for the molecule in the RC. The largest solvent effect is a 1 ppm shift for the ^{13}C 3^2 response (cf. Table 1). The reconstitution shift of 1−2 ppm detected for the 3^2 of the [U-^{13}C] in the RC may be real since the ring V keto oxygens of the accessory BChls are in close proximity. In addition, Lubitz *et al*. (1989) obtained evidence for a protein-induced rotation of the Φ_A vinyl group in PSII reaction centers, which may also occur in the reconstituted bacterial RC. Interestingly, there is no evidence for asymmetries between the two pheophytins in the vinylic region, for instance, due to an effect of the phenolic OH of Tyr M210 on the Φ_A vinyl group, which is located at a distance of 4.53 Å of the 3^1 of the BPheo in the native RCs (Ermler *et al*., 1994).

Finally, the tentative assignments for the 13 and the 13^2 suggest that ring V of one or both Pheo interacts weakly with the protein. In this respect, in the native system, the ring V of the BPheo on the L side is close to the Trp L100 amino acid residue with the 13^2, at a distance of 2.89 Å from the indole N, while the distance to the Glu L104 carboxyl side chain is 3.49 Å. On the M side, the distance between the indole nitrogen of Trp M129 and $C13^2$ is also short, 2.81 Å (Ermler *et al*., 1994).

CONCLUSION

To probe protein−pheophytin interactions in the bacterial RC to atomic resolution with CP/MAS NMR, 1-D and 2-D correlation data were collected from RC reconstituted with [U-^{13}C]Pheo *a* that was prepared biosynthetically. Pheophytin signals were assigned and used to study the electronic structure, polarization, and protonation/hydrogen-bonding state. According to the NMR results, only weak electrostatic interactions between the Pheo *a* and the surrounding protein complex occur, affecting the electronic ground state of the Pheo *a* in the RCs only moderately. Evidence for weak protein−Pheo *a* interactions at the level of ^{13}C shifts modified by binding to the protein is at the present level of the technology restricted to the chain of ring IV and possibly ring V. In contrast, there is no evidence for a selective perturbation of one Pheo *a* by the Glu L104. Finally, the NMR data indicate a great deal of similarity between the two Pheo *a* in terms of local atomic charge densities for ring carbons, which is of interest in view of the asymmetry of the primary charge separation processes. Further improvement of this novel multispin labeling and 2-D NMR approach for the characterization of protein−ligand interactions to atomic resolution will be obtained when dedicated ultra-high-field MAS equipment presently under development will become available.

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