The electronic structure of the primary electron donor of reaction centers of purple bacteria at atomic resolution as observed by photo-CIDNP $^{13}$C NMR

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Composed of the two bacteriochlorophyll cofactors, PL and PM, the special pair functions as the primary electron donor in bacterial reaction centers of purple bacteria of *Rhodobacter sphaeroides*. Under light absorption, an electron is transferred to a bacterio- pheophytin and a radical pair is produced. The occurrence of the radical pair is linked to the production of enhanced nuclear polarization called photochemically induced dynamic nuclear polarization (photo-CIDNP). This effect can be used to study the electronic structure of the special pair at atomic resolution by detection of the strongly enhanced nuclear polarization with laser-flash photo-CIDNP magic-angle spinning (MAS) NMR on the carotenoid-less mutant R26. In the electronic ground state, PL is strongly disturbed, carrying a slightly negative charge. In the radical cation state, the ratio of total electron spin densities between PL and PM is 2:1, although it is 2.5:1 for the pyrrole carbons, 2.2:1 for all porphyrin carbons, and 4:1 for the pyrrole nitrogen. It is shown that the symmetry break between the electronic structures in the electronic ground state and in the radical cation state is an intrinsic property of the special pair supermolecule, which is particularly attributable to a modification of the structure of PL. The significant difference in electron density distribution between the ground and radical cation states is explained by an electric polarization effect of the nearby histidine.

electron transfer | nuclear polarization | photosynthesis | solid-state NMR | electronic structure

The essential steps in photosynthesis, photon absorption, and electron transfer occur in the reaction center (RC) membrane protein. Simple purple photosynthetic bacteria possess only a single type of RC and perform anoxygenic photosynthesis. In RCs from the purple bacterium *Rhodobacter sphaeroides* R26, the primary electron donor (P), called the special pair, consists of two symmetrically arranged BChl $a$ (Fig. L4) cofactors, labeled PL and PM, coordinated by His-L168 and His-M202, respectively (Fig. 1B) (1, 2). The other cofactors are two accessory BChls, two BPhes $a$, two ubiquinones, and a nonheme iron that are arranged in a nearly $C_2$ symmetry. Despite the asymmetrical arrangement, the electron pathway is entirely unidirectional, occurring along the L branch (for review, see ref. 3).

In the dark electronic ground state, the symmetry between both cofactors PL and PM is already broken, as was shown with photochemically induced dynamic nuclear polarization (photo-CIDNP) $^{13}$C magic-angle spinning (MAS) NMR (4). The ratio of electron spin densities between the two cofactors in the radical cation state has been determined at the molecular level to be $\approx$2:1 using $^1$H electron nuclear double resonance (ENDOR) (5, 6) and steady-state photo-CIDNP $^{13}$C MAS NMR (7). On the other hand, values of $\approx$5:1 have been observed by $^{15}$N-ESEEM (ESEEM, electron spin echo envelope modulation) (8) and values of $\approx$4:1 have been observed by time-resolved photo-CIDNP $^{15}$N MAS NMR (9). It has been proposed that this difference is caused by distinct conformations of the RC (8) or by the different positions of the nuclei observed (9). As measured from the CH$_3$ hyperfine couplings at positions 2$^1$ and 12$^1$ by $^1$H ENDOR, mutant RCs (10, 11) have ratios of electron spin densities between $\approx$5:1 and $\approx$1:5. Detergents have also been shown to redistribute electron spin densities significantly (12).

To understand the origin of this experimentally observed variation in electron spin density distribution, the present work aims to zoom into the electronic structure of the special pair from the molecular resolution to the atomic resolution. To that end, photo-CIDNP, which occurs in frozen and quinone-removed RCs, was studied by MAS NMR. The origin of the solid-state photo-CIDNP effect in frozen and quinone-blocked RCs of *Rb. sphaeroides* R26 at high magnetic fields (13–16) is now understood (17); for reviews, see the publications by Matysik and Jeschke (14) and Daviso et al. (18).

A single photocycle is presented in Fig. 2. From the photoc- chemically excited P*, an electron is transferred to the primary acceptor. This light-induced spin-correlated radical pair is created in a nonstationary and highly electron polarized singlet state. The radical pair undergoes two processes:

(i) Intersystem crossing (ISC) driven by the isotropic hyperfine iteration $\Delta h_{iso}$ and the difference in g values of the electrons leads to a coherent oscillation between the singlet (S) and the triplet (T) states of P$^o$.

(ii) The singlet state is quenched by a radiationless transition (19, 20).

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 1AIJ).

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(T$_0$) states of the radical pair. The S state enriches the $\alpha$-nuclear spin state (with $^{13}$C having a positive gyromagnetic ratio), whereas the T$_0$ state enriches $\beta$-nuclear spins. Therefore, nuclear spin populations oscillating between $\alpha$- and $\beta$-spin states during ISC lead to transient nuclear polarization (TNP). In addition, the evolution of the spin system leads to three-spin mixing (TSM), which transfers the electron polarization to nuclei on the nanosecond time scale by mixing electronic and nuclear states under the action of the anisotropic hyperfine interaction ($\Delta 4$) and the electron–electron dipolar coupling ($d'$) (see SI) (19).

(ii) Electron back-transfer to the special pair can occur in the S and T$_0$ states having different lifetimes of the two decay channels (Fig. 2). This kinetic symmetry breaking, together with $\Delta 4$, creates additional nuclear polarization by the differential decay (DD) mechanism (20).

Both the TSM and DD contribute to the photo-CIDNP build-up by unbalancing the ratio of $\alpha$- to $\beta$-nuclear spins in the two decay channels. Both mechanisms require $\Delta 4$ and have been shown to explain the results of steady-state photo-CIDNP MAS NMR experiments under continuous illumination (7). According to theoretical considerations (21) and numerical simulations (17), the polarization arising at the end of the photocycle in $Rb$. sphaeroides WT from these two mechanisms is roughly proportional to $\Delta 4^2$, and thus to the square of $p$ spin density on the carbon atoms.

Under the steady-state conditions achieved with continuous illumination, excited states are not significantly populated; hence, the TNP caused by the $a_{iso}$ is not observed, because the contributions have opposite signs and the same magnitude in the two decay channels, and thus cancel on return to the ground state. Therefore, the radical pair mechanism (RPM) (22, 23), which is known from liquid photo-CIDNP NMR, cannot contribute to solid-state photo-CIDNP under continuous illumination.

On the other hand, because of development of time-resolved photo-CIDNP MAS NMR (24), the TNP can be observed selectively from the electronic S state (9, 17). In such experiments, the light pulse for excitation ($\sim$8 ns) and the NMR pulse for detection ($\sim$4 $\mu$s) are much shorter than the lifetime of the P molecular triplet state ($^{3}$P) state of R26 RCs ($\sim$100 $\mu$s) (Fig. 2). Thus, TNP of the S state can be transiently detected on an electronic ground state population that arises primarily from the singlet decay pathway, whereas nuclear spins in the triplet branch are still invisible because of paramagnetic broadening (17). Thus, effects attributable to isotropic coupling between electrons and nuclei become observable, similar to RPM-based photo-CIDNP in liquids. The sign rules are the same as for cage products from a singlet-born pair in RPM-based photo-CIDNP (25). Therefore, the time-resolved experiment can provide estimates of the $a_{iso}$ (vide infra).

In this work, the experimentally directly accessible TNP intensities are used to reconstruct the electronic structure of the special pair in its radical cation state at atomic resolution. The strong signal enhancement allows for assignment of the associated $^{13}$C chemical shifts (CSS) and subsequent reconstruction of the ground state electronic structure.

Results

Time-Resolved $^{13}$C Photo-CIDNP MAS NMR. Fig. 3 shows the $^{13}$C MAS NMR spectra of RCs of $Rb$. sphaeroides R26 collected in the dark (spectrum 3A) and in a time-resolved experiment in which light excitation was followed by an NMR detection pulse of 4 $\mu$s with a delay of 0 $\mu$s, with this being a time scale much shorter than the triplet donor lifetime (spectrum 3B) and that of 400 $\mu$s (spectrum 3C). Spectrum 3A shows no signal, demonstrating that all signals in spectra 3B and 3C are light-induced. Spectrum 3B presents the TNP occurring in the ground state from the singlet decay branch only. Spectrum 3C, representing the full photo-CIDNP build-up after one photocycle, agrees within the noise level of the spectrum obtained in the steady state under continuous illumination (26). Spectra 3B and 3C are distinguished, for example, in the aliphatic region (between 0 and 55 ppm), where positive (20–35 ppm) and negative (45–55 ppm)
ppm) signals appear only transiently, and in the aromatic region, where a transient negative feature at 120–135 ppm emerges.

**CS Assignments by $^{13}$C photo-CIDNP MAS NMR.** Spectrum 3B has been analyzed with fitting Lorentzians (Fig. S1). These fits were based on $^{13}$C chemical shift (CS) assignments of the P obtained in previous studies (4, 7, 26, 27) (Tables S1 and S2). For reconstruction of the ground state electronic structure, the empirically obtained CSs have been corrected for ring-current-induced shifts (Tables S1 and S2). Based on these assignments, maps at atomic resolution can be constructed from the experimentally obtained local electron densities of the electronic ground state (Fig. 4 A and A’) as well as from the experimentally obtained local electron spin densities of the radical cation state (Fig. 4 B and B’).

**Simulation of TNP Intensities.** Based on the x-ray structure of the R26-RC by Stowell et al. (28), the isotropic hyperfine interaction (hfi) values of the $a_{\text{iso}}$ have also been obtained from s spin densities on the carbon atoms of the radical cation of the P by density functional theory (DFT) calculations (Figs. S2 and S3, Tables S5 and S6). Based on the theory, TNP intensities have been computed, and with the CS assignments, spectrum 3D has been simulated assuming that the photo-CIDNP intensities are proportional to the $a_{\text{iso}}$. The general good agreement between experiment and simulation supports the theoretical prediction (see SI Materials and Methods) that the polarization arising from the S state is indeed proportional to the $a_{\text{iso}}$. Further support is obtained by simulating coupled-spin dynamics and decay kinetics of the radical pair with a density matrix approach and correlating the computed TNP polarizations originating from singlet pairs with the $a_{\text{iso}}$ values used in the input spin Hamiltonian. We find linear correlation with a correlation coefficient of 0.8779. Hence, to a good approximation, ratios of intensities of the spectral lines in the photo-CIDNP spectrum obtained at a delay time of 0 μs with a 4-μs NMR detection pulse are proportional to ratios of the corresponding $a_{\text{iso}}$ (Fig. 3B, Tables S1 and S2). Except for effects of electron–electron correlation in the wave function, $a_{\text{iso}}$, in turn, is proportional to the s spin density of the nucleus under consideration, which is obtained by representing the wave function as a linear combination of atomic orbitals. Relative spectral line intensities can thus be used to reconstruct a map of the electron spin density distribution of the special pair (Fig. 4 C and C’).

**Discussion**

**Electron Density Aberrations in the Ground State of the P.** The influence of interactions between the two chromophores and of the scaffolding protein on the electron density distribution in the electronic ground state of the special pair can be estimated from the difference in the $^{13}$C CSs between photo-CIDNP MAS NMR data of the special pair and NMR data of a BChl molecule in solution in acetone (29, 30). In Fig. 4 A and A’, the yellow spheres represent up-field shifts, which are caused by an increase of local electron density, whereas the orange spheres represent down-field shifts (see SI Materials and Methods). The prevalence of up-field shifts indicates that the total electron density in the ground state is increased compared with that of a BChl molecule in acetone solution, suggesting that the special pair carries a partial negative charge induced by the protein, presumably via hydrogen-bonding interactions via the His-L173 coordinated with the oxygen of the C-3’ acetyl of PL. The electron density is significantly increased in the overlapping region of the cofactors formed by the two pyrrole rings I, particularly on cofactor PtI. This distribution enhances interaction between the two moieties of the special pair, thus lowering excitation energy and making this pair a better energy sink for excitation transfer from light-harvesting pigments.

The CSs are significantly different between the two parts of the special pair (Tables S1 and S2), as proposed earlier by Schulten et al. (4). The concentration of electron density of pyrrole ring I of cofactor PtI is linked to up-field shifts of up to 14 ppm, which cannot be explained by ring-current shifts (Tables S1 and S2). The high electron density of both pyrrole rings I may be stabilized by the hydrogen bond interaction between the protein and the special BChl α of the special pair. The existence of this hydrogen bond (31–33) and its crucial role for the
Electron Spin Densities in the Radical Cation State. Fig. 4 B and B* show the map of $^{13}$C photo-CIDNP intensities of PL and PM in the radical cation state obtained from the spectrum collected by a light pulse followed by an NMR detection pulse much shorter than the lifetime of the triplet $^3$P (vide infra).

The size of the spheres is proportional to the magnitude of the $a_{\text{iso}}$. The red spheres represent positive (absorptive) NMR signals associated with an excess of $\alpha$-nuclear spin states, whereas the blue spheres are associated with negative (emissive) signals attributable to excess of $\beta$-nuclear spin states. According to RPM sign rules that also apply to TNP, absorptive and emissive signals are associated with positive and negative $a_{\text{iso}}$, respectively. The size of the spheres has been normalized on the most intense signal arising from C-9 of PL (161 ppm). The ratio of the total electron spin densities in $s$ orbitals on the detected carbon atoms between both cofactors is $(68 \pm 4):(32 \pm 4)$ in favor of PL (Tables S1 and S2).

Hence, the molecular electron spin density distribution is in very good agreement with previous $^1$H ENDOR studies (6), which inferred carbon spin densities from the $a_{\text{iso}}$ of directly bonded protons via McConnell relations, and with steady-state $^{13}$C-photo-CIDNP studies (7), which are sensitive to the spin densities in the carbon $2p_z$ orbitals that dominate the anisotropic contribution to the $^{13}$C hyperfine coupling.

Zooming into the atomic scale, the electron spin density is localized mainly on the periphery of the special pair, whereas the central overlapping region appears to be emptied. It seems that the supramolecular structure now stabilizes the hole.

In addition to the supramolecular asymmetry (PL over PM) and the molecular asymmetry (pyrrole rings II and IV over rings I and III), a symmetry break occurs on the level of the pyrrole rings. Particularly high electron spin density is observed on the periphery of the special pair, whereas the coupling. In particular, the phase patterns of electron spin densities residing on the eight nitrogens, a ratio of 5:2 in favor of PL occurs (see SI Materials and Methods).

The alternating phase pattern found by the calculations (Fig. 4 C and C*) exactly matches the one observed spectroscopically (Fig. 4 B and B*). In addition, the electron spin density ratio between PL and PM is in good agreement between theory and experiment. The successful theoretical simulation implies that the asymmetry in the radical cation is an intrinsic property of the special pair complex with its axial histidines and is caused by geometrical differences conserved by the matrix. Hence, the matrix effect is predominantly attributable to local conformational conservation and caused neither by a far-reaching Coulomb interaction of charged amino acids nor by overlap with other cofactors or aromatic amino acids. In other words, the protein matrix induces the asymmetry by imposing special chlorophyll geometries rather than by delocalization of the wave function beyond the chlorophylls. Interestingly, the matrix has been shown to be extremely rigid and without structural heterogeneities (37, 38). As already assumed by Diller et al. (39) on the basis of photo-CIDNP MAS NMR on photosystem II, cofactor–matrix units of functional relevance are conserved in evolution. That principle may explain the observation by Shelnut et al. (40) on porphins that cofactor geometries are related to their function.

The maximum close to the primary acceptor may be related to locally enhanced orbital coefficients facilitating electron transfer into the L branch. It appears that the special properties of PL, which are well reflected in the calculations, are an inherent function of the special pair supermolecule.

Comparison of Electronic Structures in Ground and Radical Cation States. The local electron density of the special pair in its electronic ground state has the maximum in the inner part of the supramolecule, in the overlapping region of the two pyrrole rings I, whereas the electron spin density map of the radical pair state shows a clear and significant asymmetry toward the primary electron acceptor in the active branch. Remarkably, both maxima are found in the special BChl cofactor (P$_L$).

Both electronic structures elucidated, that of the electronic ground state as well as that of the radical cation state, reflect the same molecular orbital that is the HOMO of the special pair. However, within the static model of the Marcus theory, the differences between the two electronic structures are unexpected. Therefore, the significant differences must reflect the effects of the surrounding of the highest occupied molecular orbital (HOMO). Hence, we conclude that the matrix undergoes local electric polarization effects. Such an local electric polarization effect of the matrix is consistent with and lends support to the previous assumption that the hydrogen bond between the C-3′ acetyl of P$_L$ and His-L173 stabilizes the charge in the central overlapping region of the special pair in its electronic ground state, although it is broken or weakened in the radical cation state (11, 34). This, in turn, may locally change the dielectric constant of the protein matrix and may help to prevent the back-electron transfer.

Materials and Methods

Sample Preparation. The RCs from Rb. sphaeroides R26 were isolated by the procedure of Feher and Okamura (41). The removal of the quinones has been done by incubating the RCs at a concentration of 0.6 $\mu$g/ml in 4% (vol/vol) lauryldimethylamine-oxide (LDAO), 10 mM o-phenanthroline, 10 mM Tris buffer, pH 8.0, for 6 h at 25 °C, followed by washing with 0.5 M NaCl in 10 mM Tris buffer, pH 8.0, containing 0.025% LDAO and 1 mM EDTA (42). Double-approximately 5 mg of the RC protein complex embedded in LDAO micelles was used for NMR measurements.
MAS NMR Experiments. NMR experiments were performed with an Avance-200 NMR spectrometer equipped with a MAS probe (Bruker). The sample was loaded into a 4-mm sapphire rotor and inserted into the MAS probe, and it was frozen slowly at a low-spinning frequency of 800 Hz to ensure a homogeneous sample distribution against the rotor wall (37). The light and dark spectra were collected with a spin-echo pulse sequence with the cyclically ordered phase sequence (CYPLOPS) phase cycle of the (n2) pulse and detection under two pulse-phase modulation carbon-proton decoupling (43) at a temperature of 40 °Celsius. The optimum length of the (n2) carbon pulse, determined on uniformly 13C-labeled tyrosine, was −4 µs under our experimental conditions using a rf power of −250 W.

A pulsed nanosecond flash laser provides sufficient radiation intensity for time-resolved photo-CIDNP MAS NMR studies and does not decrease the time resolution that can be obtained in NMR experiments. The laser was operated with repetition rates between 1 and 4 Hz. Using 1,064-nm flashes of a neodymium yttrium-aluminum garnet (Nd:YAG) laser (Quanta-Ray INDI 40–10; SpectraPhysics), on frequency-doubling with a second harmonic generator, 532-nm laser flashes with pulse lengths of 6–8 ns and energy between 20 and 150 mJ were produced.

Time-resolved photo-CIDNP MAS NMR data were acquired using a presaturation pulse sequence to erase polarization and coherence from previous scans as described by Daviso et al. (24). The delay times between light excitation and NMR detection used were 0 and 400 µs. The rotational frequency for MAS was 8 kHz in all experiments. A cycle delay of 133 ms was used. All the 13C MAS NMR spectra were referenced to the 13COOH response of solid tyrosine at 172.1 ppm.

Spectral Fitting. The fitting of the spectrum collected during time-resolved MAS NMR photo-CIDNP of pure RCs of Rb. sphaeroides R26 has been performed using the program nlopt (1). The peaks are Lorentzian with a full width at half height (FWHH) between 40 and 60 Hz as experimentally observed using the 4-ALA labeling pattern of Rb. sphaeroides WT (24) (WaveMetrics). A typical characteristic fitting result is shown in Fig. S1.

DFT Calculations of the Radical Cation of the P. DFT computations of hyperfine coupling tensors were performed with the Amsterdam density functional program (AM1) 2000 package (SCM NW), using the triple-Zeta basis set with polarization functions on all atoms (TZ2P) all-electron basis set for all atoms as described (17, 26). Geometries of ground state molecules were taken from the crystal structure in the charge-neutral state [Protein Data Bank (PDB) ID code 1AU] (28) and subjected to geometry optimization within ADF in the cation radical state. Details are given in SI Materials and Methods.

Numerical Simulations of TNP Intensities. Numerical simulations start from a pure S and compute the time evolution under a Hamiltonian, including electron Zeeman, nuclear Zeeman, and hfi as well as dipole–dipole and exchange coupling between the two electron spins. The part of the density matrix that decays to the ground state from either the S or T1 state is projected out (diamagnetic part) and is further evolved under a Hamiltonian, including only the nuclear Zeeman interaction. Details are given in SI Materials and Methods.

Calculations of Ring-Current Shifts. To estimate ring-current shifts, we calculated nucleus-independent C5s (44) at the positions of the nuclei of monomer P85 in a calculation on monomer P3 or vice versa, with the NMR module (45) of ADF code 1AU (28). The dimer of BCHl molecules, the axial histidine ligands, and the histidine ligand coordinated to the acetyl group at the C3 position of P3 were extracted from that structure. Hydrogen atoms were added, and their positions were relaxed in a BP86/TZP calculation employing an empirical dispersion correction (50). The phytanyl chains of the BCHl molecules were replaced by methyl groups. All histidine ligands were assumed to be neutral.

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