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Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1767 (2007) 610–615
Biochimica et Biophysica Acta
BBA
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Photochemically induced dynamic nuclear polarization in the reaction center of the green sulphur bacterium *Chlorobium tepidum* observed by ^{13}C MAS NMR

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Received 22 September 2006; received in revised form 21 December 2006; accepted 29 December 2006
Available online 9 January 2007

Abstract

Photochemically induced dynamic nuclear polarization has been observed in reaction centres of the green sulphur bacterium *Chlorobium tepidum* by ^{13}C magic-angle spinning solid-state NMR under continuous illumination with white light. An almost complete set of chemical shifts of the aromatic ring carbons of a BChl *a* molecule has been obtained. All light-induced ^{13}C NMR signals appear to be emissive, which is similar to the pattern observed in the reaction centers of plant photosystem I and purple bacterial reaction centres of *Rhodobacter sphaeroides* wild type. The donor in RCs of green sulfur bacteria clearly differs from the substantially asymmetric special pair of purple bacteria and appears to be similar to the more symmetric donor of photosystem I.

Keywords: Green sulphur bacteria; Photosystem I; Solid-state NMR; Photo-CIDNP; Reaction centres

1. Introduction

Photosynthesis is the process in which light energy is transformed into chemical energy and stored by an organism [1]. Photosynthetic reaction centers (RCs) are classified into two types on the basis of their early electron acceptors [2–4]. The RCs containing membrane bound iron–sulphur centers are called 'Fe–S type RC' (Type I), while those containing (bacterio) pheophytin and quinones as 'pheophytin–quinone type RC' (Type II). A wide variety of photosynthetic organisms ranging from prokaryotes to eukaryotes are found. Type-I RCs are found in green sulphur bacteria, heliobacteria, cyanobacteria as well as in plants. On the other hand, type-II RCs are found in purple bacteria, cyanobacteria and in plants. Oxygenic photosynthetic organisms, such as higher plants, algae and cyanobacteria, contain both types of photosystems, namely photosystem I (PSI) and photosystem II (PSII). The two photosystems have very different redox potential properties. PSII provides a strong positive redox potential, which enables the oxidation of water and production of molecular oxygen, while PSI generates a strong negative redox potential which drives the electrons to ferredoxin, leading to the reduction of $NADP^+$ to NADPH. The question of what are the determining factors of the redox properties has recently been addressed [5–8].

Anoxygenic photosynthetic organisms are bacteria that contain a single photosystem, either type-I RCs, as found in green sulphur bacteria and heliobacteria, or type-II RCs, in purple and filamentous green bacteria. Green sulphur bacteria have large light-harvesting antenna complexes known as chlorosomes, which contain bacteriochlorophyll aggregates [9] and Fenna–Mathews–Olson (FMO) proteins [10].

Interestingly, in green sulphur bacteria and in heliobacteria a single gene of the RC core protein has been identified [11,12]. Structural analysis of the RC core complex of the green sulphur bacteria *Chlorobium (C.) tepidum* indicated the presence of a

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0005-2728/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2006.12.012

homodimer formed by two 82 kDa PscA proteins [13], in contrast to a heterodimer formed by PsaA and PsaB in PSI. A single PscA protein contains eight bacteriochlorophylls, two plant Chl *a* derivatives and between two and eleven carotenoids have been reported per RC [14,15] which is considerably less than the number of chlorophylls found attached to the heterodimeric core of PSI. Until now, no X-ray crystal structure of a RC of green sulphur bacteria has been reported.

The primary donor in the RC of green sulphur bacteria is termed P840, due to the absorption maximum at 840 nm. It has been assigned to two BChl *a* molecules [16,17], probably two C-13^2 epimers [18]. The RC of green sulphur bacteria also contains a plant Chl *a*, called Chl 670, presumably acting at the primary electron acceptor (A_0) [19]. That Chl *a* cofactor, however, is esterified with Δ2,6-pytadienol, rather than phytol as in plants and cyanobacteria [18]. Based on EPR experiments, a menaquinone cofactor has been proposed to be the secondary electron acceptor (A_1) [20,21]. The putative quinone binding site appears to be partially conserved in PSI, green sulphur bacteria and heliobacteria [22]. It has been reported that the RCs of green sulphur bacteria and heliobacteria are active without the presence of quinones [23,24]. The terminal electron acceptors are three iron sulphur centers, F_X, F_A and F_B, as detected by EPR studies on the RCs [25]. Various structural and functional aspects have been probed by several spectroscopic methods [26–30].

A rapidly emerging technique in the study of membrane proteins is magic-angle spinning (MAS) NMR [31,32]. The chemical shifts allow the exploration of the electronic and protonic structures in the electronic ground state. In RCs upon illumination, photochemically induced dynamic nuclear polarization (photo-CIDNP) has been observed by MAS NMR as modification of signal intensity [33], for review, see: [34,35]. Photo-CIDNP intensities are related to the local electron spin densities. In purple bacterial RCs of *Rhodobacter(Rb.) sphaeroides* wild type (WT) [36] and carotenoid less mutant R26 [37], the strongest enhancement of NMR signals observed is a factor of 10,000. Until now, photo-CIDNP has been observed in four photosynthetic systems: in purple bacterial RCs of *Rb. sphaeroides*WT [36,38], R26 [33,37,39–41], D1D2 complex of photosystem II of plants [6,8] and from PSI complex of plants [42].

Recently, it has been shown that three mechanisms can produce photo-CIDNP in solids [37]. Occurrence of two parallel mechanisms has been proposed [34,36]. The three spin mixing (TSM) [43], which relies on the coupling between two electron spins in a radical pair state, leading to enhanced polarization of the radical ions. This electron polarization is then transferred by an anisotropic hyperfine coupling to polarization of nuclear spins. The differential decay (DD) mechanism [44] also requires anisotropic hyperfine coupling, but the transfer of the pair polarization to single radical ion polarization arises from superposition of the singlet and triplet state of the radical pair and subsequent preferential decay of pairs in the triplet states. Furthermore, a third mechanism is active in systems having a long-lived triplet state of the donor, leading to the differential relaxation (DR) process [45].

It has been proposed that the occurrence of photo-CIDNP coincides with the conditions of the unparalleled efficient light induced electron transfer in natural RCs [34]. Until now, no photo-CIDNP has been reported in artificial RCs. The observation of photo-CIDNP in RCs of *C. tepidum*, reported here, allows the conclusion that all known types of natural RCs studied so far exhibit photo-CIDNP.

2. Materials and methods

2.1. Preparation

C. tepidum strain TLS were grown at light intensity of 1 kLux from incandescent lamps in a medium described by Wahlund et al. [46]. The 3FMO-RC particles of *C. tepidum* were isolated and purified using sucrose gradient centrifugation as described in ref. [47]. The purity of the FMO-RC particles was analysed by SDS gel electrophoresis (data not shown). The purified FMO-RC particles were then recovered from the sucrose gradient and dialysed against buffer containing 50 mM Tris/HCl and 10 mM sodium ascorbate (pH 8.3) for 3 h and then ultracentrifuged at 200,000×*g* for 3 h. The pellet containing the particles was dissolved in buffer containing 50 mM glycine and 0.01% Triton X-100 (pH 10.8). For photo-CIDNP studies the FMO-RC particles were reduced in the same buffer containing 50 mM sodium dithionite.

2.2. MAS NMR measurements

The NMR experiments were performed by using a DMX-200 NMR spectrometer (Bruker GmbH, Karlsruhe, Germany). The sample was loaded into an optically transparent 4 mm sapphire rotor. The sample was reduced by addition of an aqueous solution of 50 mM sodium dithionite in an oxygen-free atmosphere. Immediately following the reduction, slow freezing of the sample was performed directly in the NMR probe inside the magnet with liquid nitrogen-cooled gas under continuous illumination with white light [48]. The illumination set-up was specially designed for Bruker MAS probe [41]. Photo-CIDNP ^{13}C MAS NMR spectra were obtained at a temperature of 240 K with a spinning frequency of 8 kHz. The light and dark spectra were measured with a Hahn echo pulse sequence and two pulse phase modulation (TPPM) proton decoupling [49].

3. Results and discussion

3.1. Dark spectrum

Fig. 1 shows the ^{13}C MAS NMR spectra of natural abundance FMO-RC particles of *C. tepidum* in the dark (A) and under continuous illumination with white light (B) at a magnetic field of 4.7 T. Spectrum 1A shows the characteristic features of a ^{13}C-MAS NMR spectrum of a protein, i.e., broad responses between 0 and 50 ppm. The sharp signal at 175.7 ppm arises mainly from glycine buffer. Additional weak features of aromatic cofactors and amino acids appear between 190 and 80 ppm.

3.2. Overall spectral pattern

In spectrum 1B, obtained under illumination, several strong emissive (negative) signals appear. A total of ten centrebands has been identified. Due to photo-CIDNP, all signals appear to be strongly emissive (Table 1). These signals appear in the carbonylic region as well as in the aromatic region. The signals observed at lowest frequency arise at about 100 ppm from

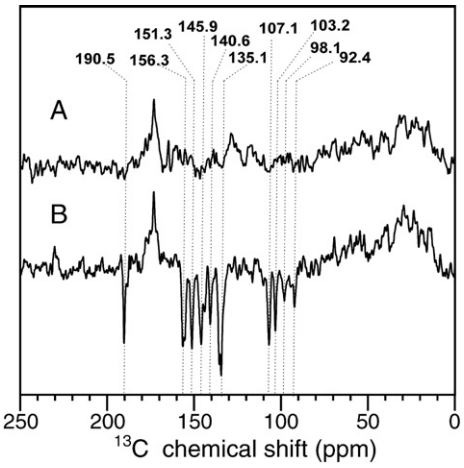

Fig. 1. ^{13}C MAS NMR spectra of RC complex of *C. tepidum* at 240 K and a MAS frequency of 8 kHz at 4.7 T. Spectra are obtained: in the dark (A) and under continuous illumination with white light (B). In both experiments, the cycle delay was 12 s and the measurement time 2 days.

methine carbons, while no photo-CIDNP is observed in the aliphatic region. This overall pattern has also been observed in RCs of PSI [42] and *Rb. sphaeroides* WT [36] and is in contrast to the pattern of positive aromatic signals combined with negative methine signals as observed in RCs of PSII [6,8] and *Rb. sphaeroides* R26 [33,37,39,40,41]. In case of the two bacterial RCs of *Rb. sphaeroides*, it has been demonstrated that the difference in the pattern is due to a difference of the lifetime of the donor triplet [37]. RCs of *Rb. sphaeroides* WT have a triplet lifetime of 100 ns, while the RCs of the carotene-less mutant R26 have a lifetime of the donor triplet of 100 μs, a time long enough to produce net polarization by the DR effect leading to an inversion of the sign of the donor signals. Hence, based on such comparison, we assume that the donor side of the RC of *C. tepidum* contains a carotene able to quench efficiently the triplet states of the donor. In fact, the presence of carotenoids in the RCs of *C. tepidum* has been reported [50] and is in-line with the observed photo-CIDNP pattern.

3.3. Assignments

Most of the signals can be assigned straightforwardly to a BChl a or Chl *a* cofactor (Table 1). In the carbonyl region, the strong and sharp signal at 190.5 ppm is detected and can be assigned directly to the carbonyl carbon C-13^1. Such strong emissive signal of a carbonyl carbon has been observed in the photo-CIDNP spectrum of PSI [42], where it has been assigned to the donor, while it is weak in the spectrum of *Rb. sphaeroides* WT [36]. The strongest signals are observed in the aromatic region between 120 and 170 ppm. The signal at 156.3 ppm may be doubled and can be assigned to C-9 of a BChl *a* or C-1 and C-6 of a Chl *a*. The peak at 151.3 ppm can be assigned to C-4 or C-16 of either a BChl *a* or Chl *a* cofactor. The signal at 145.9 ppm, having a clear shoulder on its low-frequency wing, can arise from a C-11 of a BChl *a* or from C-8 of a Chl *a*. The signal at 140.6 ppm can be assigned to C-2 of a BChl *a*, while an assignment to a Chl *a* is rather unlikely. The signal at 135.1 ppm shows a shoulder and can be assigned to C-3 of BChl *a* or C-2 of Chl *a*. Also in the region of the methine carbons, most signals may be assigned to either the BChl *a* donor molecule(s) or to the Chl *a* acceptor. The signal at 107.1 ppm can be assigned to the C-15 of a BChl *a* or a C-10 of a Chl *a*, while that at 103.1 ppm can arise from the C-10 of a BChl *a* or a C-15 of a Chl *a*. The signals at 98.1 and 92.4 ppm originate form the C-5 and C-20, respectively, from either the BChl *a* or the Chl *a*.

Hence, the chemical shift information is not sufficient to assign the photo-CIDNP signals to either the donor or the acceptor, although the strength of the carbonyl signal and the chemical shift of 140.6 ppm indicate that at least some contribution from the donor exists. In analogy to PSI and the RC of *Rb. sphaeroides* WT, in which the signals above ~130 ppm were assigned to the donor based on simulations of donor and acceptor photo-CIDNP intensities, we tend to assign the group of aromatic carbons above 130 ppm to the donor, while we have no conclusive evidence for the assignment of the signals of the methine carbons to either the donor or the acceptor.

Table 1
^{13}C chemical shifts of the photo-CIDNP signals observed in *Chlorobium tepidum* in comparison to chemical shift data of Bacteriochlorophyll *a* and Chlorophyll *a*

Chl *a*		PSI	Carbon no.	BChl *a*		*C. tepidum*
σ_{liq}[a]	σ_{ss}[b]	σ[c]		σ_{liq}[d]	σ_{ss}[e]	σ[f]
189.3	190.6	~190.6 E	**13^1**	199.3	188.2	190.5 E
172.7	175.3		**17^3**	173.4	174.0	
171.0	171.2		**13^3**	171.6	171.4	
167.4	170.0	167.1 E	**19**	167.3	168.9	
161.4	162.0	160.4 E	**14**	160.8	160.7	
154.0	155.9	154.8 E	**1**	151.2	153.5	
155.8	154.4		**6**	168.9	170.2	
151.4	154.0	152.6 E	**16**	152.2	150.1	
148.0	150.7	149.9 E	**4**	150.2	152.2	151.3 E
147.7	147.2	147.2 E	**11**	149.5	147.2	145.9 E
146.1	147.2		**9**	158.5	158.0	156.3 E
144.1	146.2	144.2 E	**8**			
139.0	137.0	138.6 E	**3**	137.7	136.1	135.1 E
135.5	136.1	~136 E	**2**	142.1	140.7	140.6 E
134.2	134.0		**12**	123.9	119.9	
134.0	133.4		**7**			
131.5	126.2	~132 E	**13**	130.5	124.1	
131.5	126.2		**3^1**	199.3	194.5	
118.9	113.4		**3^2**			
107.1	108.2	105.4 E	**10**	102.4	100.0	103.2 E
106.2	102.8		**15**	109.7	105.8	107.1 E
100.0	98.1		**5**	99.6	98.8	98.1 E
92.8	93.3		**20**	96.3	93.7	92.4 E

[a] See ref. [53]. The liquid NMR data have been obtained in tetrahydrofuran.
[b] See ref. [54]. The solid state NMR data have been obtained from aggregates.
[c] Ref. [42].
[d] Ref. [41] The liquid NMR data obtained in acetone-d_6.
[e] Ref. [55].
[f] this work.
Abbreviations: σ = chemical shift, E = emissive signal.

3.4. Line shape and line width

Some of the donor signals appear to be doubled or show a shoulder, namely the signals at 156.3, 145.9 and 135.1 ppm. Due to their chemical shift values, these signals for which we do observe splittings cannot be explained by originating from to two different carbons. This signal doubling can be interpreted in terms of a slightly asymmetric dimer. If this is the case, small differences between the two halves exist in both, the electronic ground state, indicated by the chemical shift differences and the radical cation, indicated by different signal intensities. This interpretation depends on the assignment of these signals to the donor. First, it implies that the two branches of *C. tepidum* RCs differ much less from each other than in RCs of purple bacteria, where a clear asymmetry in the electronic ground state has been demonstrated for the special pair donor [36,38,51,52] and the radical cation state [36]. This is hardly surprising, as *C. tepidum* RCs appear to be scaffolded by a protein homodimer, while a heterodimer is found in purple bacteria. The slight asymmetry, however, indicates that the two branches are not fully equivalent, which in turn implies that the symmetry of the homodimer is broken by either interactions with neighboring molecules or posttranslational modification of one half.

^{15}N photo-CIDNP MAS NMR data, very recently obtained in our laboratory (A. Diller et al., unpublished data) suggest also for the donor of PSI a slightly asymmetric dimer. The quality of the ^{13}C photo-CIDNP MAS NMR data of PSI, however, does not allow for a safe differentiation between a slightly asymmetric dimer and a monomer.

The five signals at 190.5, 151.3, 140.6, 103.2 and 107.1 ppm do not indicate any doubling and appear to be remarkably narrow, as indicated by full width at half-height (FWHH) of 54.1, 68.9, 64.0, 56.6 and 73.8 Hz, respectively. These line widths are similar to those found in PSI [42], reveal a rigid, ordered as well as structurally and electrostatically stable donor side, keeping the reorganization energies of the electron transfer low. Hence, the donor of the RC of *C. tepidum* is probably similar in electronic structure and rigidity to that of PSI, despite of the difference in the chemical structure of the cofactors.

3.5. Results of other spectroscopic methods

Data on RCs of *C. limicola* obtained by ENDOR and Special TRIPLE spectroscopies show that $P840^{+\cdot}$ has a symmetrical distribution over the two halves of the pair, having approximately a 1:1 distribution of electron spin density [27]. This conclusion on the radical-pair state matches with our observation of similar photo-CIDNP intensities of both parts of split signals, making an interpretation of an asymmetric dimer $P840^{+\cdot}$ unlikely [30]. On the other hand, circular dichroism data on RCs of *C. tepidum* were interpreted in terms of a difference in asymmetry of the P840 donor relative to the special pair in purple bacteria [29]. Our chemical shift data do not allow for an interpretation of a strong asymmetry within the P840 donor dimer in the ground state. That is in contrast to the special pair of RCs of purple bacteria, were the symmetry is already broken in the electronic ground state [36,38,51,52]. Hence, the difference observed by CD spectroscopy may be caused by differences occurring in the electronic ground-state. FTIR data on the primary donor have shown that at least one of the two BChl *a* forming the primary donor is free from hydrogen bonding [30]. Due to the similarity of chemical shifts it is suggested that both BChl cofactors of P840 are in the same hydrogen bounding state.

4. Conclusions

Photo-CIDNP has been observed in RCs of the green sulphur bacterium *C. tepidum*. It appears that photo-CIDNP is an inherent property of all types of natural RCs. In the ^{13}C photo-CIDNP MAS NMR spectrum of the RC of *C. tepidum*, all signals are emissive (negative). The overall photo-CIDNP pattern is similar to that observed in PSI. The carbonylic and aromatic signals can be assigned to the two BChl *a* molecules of the donor side. Doubling of several signals suggests an only slightly asymmetric dimer in both the electronic ground-state and radical-cation state of the donor side. Hence, the donor in RCs of green sulfur bacteria clearly differs from the substantially asymmetric special pair of purple bacteria and appears to be similar to the more symmetric donor of PSI.

Acknowledgements

We thank Mr. A.H.M. de Wit for growing the bacteria. The help F. Lefeber and J.G. Hollander and K. Erkelens for providing technical support is gratefully acknowledged. This work has been financially supported by the Netherlands Organization for Scientific Research (NWO) through Jonge Chemici award (700.50.521), an open competition grant (700.50.004) and a Vidi grant (700.53.423) as well of the Volkswagen-Stiftung (I/78010) to JM.

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