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Heteronuclear 2D (^{1}H-^{13}C) MAS NMR resolves the electronic structure of coordinated histidines in light-harvesting complex II: Assessment of charge transfer and electronic delocalization effect

Alia[a], Jörg Matysik[a], Ido de Boer[a], Peter Gast[b], Hans J. van Gorkom[b] & Huub J.M. de Groot[a,*]

[a]*Leiden Institute of Chemistry, Gorlaeus Laboratoria, P.O. Box 9502, 2300 RA Leiden, The Netherlands;*
[b]*Department of Biophysics, Huygens Laboratorium, P.O. Box 9504, 2300 RA Leiden, The Netherlands*

Abstract

In a recent MAS NMR study, two types of histidine residues in the light-harvesting complex II (LH2) of *Rhodopseudomonas acidophila* were resolved: Type 1 (neutral) and Type 2 (positively charged) (Alia et al. *J. Am. Chem. Soc.*). The isotropic ^{13}C shifts of histidines coordinating to B850 BChl *a* are similar to fully positively charged histidine, while the ^{15}N shift anisotropy shows a predominantly neutral character. In addition the possibility that the ring currents are quenched by overlap in the superstructure of the complete ring of 18 B850 molecules in the LH2 complex could not be excluded. In the present work, by using two-dimensional heteronuclear (^{1}H-^{13}C) dipolar correlation spectroscopy with phase-modulated Lee–Goldburg homonuclear ^{1}H decoupling applied during the t_1 period, a clear and unambiguous assignment of the protons of histidine interacting with the magnesium of a BChl *a* molecule is obtained and a significant ring current effect from B850 on the coordinating histidine is resolved. Using the ring current shift on ^{1}H, we refine the ^{13}C chemical shift assignment of the coordinating histidine and clearly distinguish the electronic structure of coordinating histidines from that of fully positively charged histidine. The DFT calculations corroborate that the coordinating histidines carry $\sim$0.2 electronic equivalent of positive charge in LH2. In addition, the data indicate that the ground state electronic structures of individual BChl *a*/His complexes is largely independent of supermolecular π interactions in the assembly of 18 B850 ring in LH2.

Introduction

Histidine (His) residues play an important role in biocatalytic molecular processes of many proteins. Their imidazole side chains can occur in different protonation and charge states and form hydrogen bonds to the surroundings. Interaction of histidine with Mg^{2+} has been suggested in all BChl-protein complexes with known structures (Prince et al., 1997; Matthews et al., 1997; Deisenhofer et al., 1985).

Although histidine residues are the main ligands to B(Chl) in all known photosynthetic reaction centres, and many antenna complexes, detailed knowledge

about the protonation and electronic state of these residues is still lacking. High resolution solid-state NMR in combination with isotope labelling provides clear access to the local spatial and electronic structure around amino acid side chains in large membrane protein complexes (Castellani et al., 2002; Egorova-Zachernyuk et al., 2001). Using MAS/NMR studies in conjunction with site-directed isotope labelling, we provided recently a conclusive ^{15}N chemical shift assignment of histidine nitrogens coordinating with Mg^{2+} in the light-harvesting complex II (LH2) of *Rhodopseudomonas (Rps.) acidophila* (Alia et al., 2001). LH2 is a peripheral antenna complex that serves to absorb light and to transfer the excited state energy to the LH1-reaction centre complex. The high

resolution X-ray structure of LH2 complex of *Rps. acidophila,* showed a remarkable symmetry in the arrangement of light absorbing pigments in their protein matrix (McDermott et al., 1995; Cogdell et al., 1999). The whole complex is an oligomer of 9 identical units arranged in a ring. Each unit consists of a pair of small hydrophobic apoproteins (named α and β), a pair of BChl *a* molecules absorbing at 850 nm (B850) and one BChl *a* molecule absorbing at 800 nm (B800) (Figure 1). The 18 B850 molecules form a closely interacting ring. Going around the ring of B850, the Mg^{2+} ions are coordinated alternatively to the His30 on β apoprotein (β-His30) and then to His31 on the α apoprotein (α-His31) (Figure 1). These two histidines are highly conserved in different bacteria. This overall ring assembly of B850 acts as an energy storage device, preserving excitation energy until it is forwarded to other rings and ultimately to the RC (Hu and Schulten et al., 1997; Hu et al., 1998). The exact mode of energy storage, however, is not yet fully understood. Once the ring of B850 is excited, a depolarisation is observed in 200 fs. This suggests that the energy is either delocalized extensively (van Oijen et al., 1999) or rapidly migrates around the ring. How far the delocalization progresses is a subject of debate (Cogdell et al., 1999). In order to understand such a system information on the electronic coupling between molecules, the coupling to the protein and the nature and magnitude of the electronic couplings are needed (Zhang et al., 1998; Scholes et al., 1999). The two conserved histidines β-His30 and α-His31 mediate the short range coupling between the B850 and stabilise the B850 ring assembly in the LH2 complex (Scholes et al., 1999). Understanding of the electronic structure of His/B850 complex is thus important for understanding the mechanism of exciton transfer over the BChl ring in LH2.

By means of site specific ^{15}N labelling, we recently demonstrated that the τ nitrogen of β-His30 and α-His31 ligate to the Mg^{2+} of the B850 BChl *a* molecules, while the the π nitrogen of these histidines may be hydrogen bonded to the 9 keto group of the adjacent B850 (Alia et al., 2001). The apoproteins α contain two histidine (α-His31 and α-His37) while apoproteins β contain three histidines (β-His12, β-His30 and β-His41). By using 2-D MAS NMR homonuclear ([13]C-^{13}C) and double CP/MAS experiments, two different sets of chemical shifts for the histidines in the LH2 have been resolved (Alia et al., 2001). However, the interpretation of these sets in terms of the electronic structures of the various his-

tidine side chains is not yet complete. The first set (Type 1) corresponds with the neutral form of histidine (β-His12 and α-His37) (Scheme 1). Surprisingly, for the second species (Type 2) the carbon chemical shifts of the two histidines bound to the Mg^{2+} (β-His30, α-His31) are similar to those of fully positively charged histidine (β-His41). In contrast, the anisotropy parameters suggested that the N...Mg type nitrogen has an electronic structure similar to that of the lone pair type nitrogen (Alia et al., 2001). This led us to propose a partial positive charge character due to the presence of a proton on the π-nitrogen and Mg on the τ-nitrogen in an overall neutral BChl*a*/His complex. This raised the question if carbons of β-His30 and α-His31 experience a ring current shift due to their close proximity to the aromatic ring of the B850. The extensive delocalization of the electrons in the porphyrin ring of B850 should produce relatively large shifts on the carbons of the coordinated histidine. It was presumed that the ring currents might have been affected by the overlap in the superstructure of the complete ring of 18 B850 molecules. In addition, the assignment of ε carbon of the Type 1 and Type 2 histidines was not in line with the values obtained with model studies. According to model studies, ε of Type 2 histidines is expected to resonate 2-3 ppm downfield from the ε for Type 1 histidines, but in LH2 we could not distinguish between the ε carbon of Type 1 and Type 2 histidines (Alia et al., 2001).

In the present study, using [1]H-^{13}C MAS HETCOR experiments on LH2, we assess the charge transfer and electronic delocalization effect on coordinating histidines in LH2. This is the first time that a clear and unambiguous assignment of the proton and ^{13}C resonances of the imidazole side chains of the histidines interacting with magnesium of BChl *a* molecules is made for an intrinsic membrane protein. Ring current effects are much more pronounced in ^{1}H NMR than in ^{13}C NMR due to the smaller chemical shift range for the [1]H. A significant ring current effect on the protons of β-His30 and α-His31 from B850 was observed. On the basis of the [1]H-^{13}C HETCOR data we are able to clearly distinguish the ^{13}C response of the histidines interacting with B850 from the signals associated with the fully positively charged histidines. This demonstrates that the Mg-bound histidines have a different electronic structure compared to the fully positively charged β-His41. Our results show that the supermolecular π − π interactions in the assembly of 18 B850 ring in LH2 are moderate, since they do not
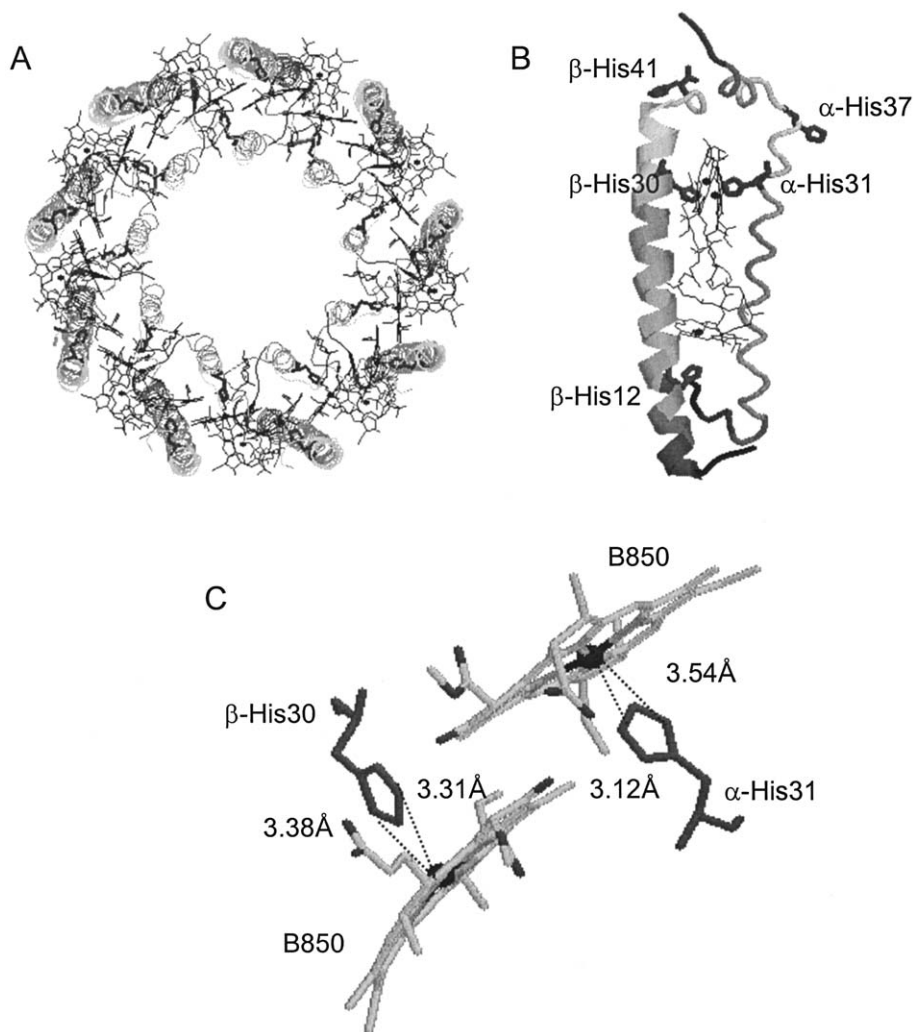
Figure 1. Arrangement of histidines in LH2 of *Rps.acidophila*. The helices are represented by ribbons. (A) Top view; (B) side view of one of the protomers of LH2; (C) a portion of the ring showing distances between the δ and ε carbons of β-His 30 and α-His 31 and the central Mg atoms of coordinated B850 molecules. The aliphatic chains of BChl have been omitted for clarity.

quench the ring currents for the individual BChl *a*/His complexes.

Materials and methods

Culturing of Rps. acidophila *and incorporation of* $[^{13}C_6, ^{15}N_3]$-L-*histidine*

Rps. acidophila strain 10050 was grown anaerobically at 30 °C in sterile liquid medium. For each liter of culture medium, 20 ml of 1.0 M ammonium malate solution (pH 6.9), 20 ml of 1.0 M phosphate buffer (pH 6.9), 20 ml of trace elements, 4 μl of a solution of thiamine (25 g l^{-1}) and biotine (0.5 g l^{-1}) was added. The trace elements were prepared as described by Raap et al. (1990). The pH of the medium was adjusted to 5.8. The cultures were allowed to grow for 7 days in light (2000 lux provided by incandescent lamps).

For incorporation of $[^{13}C_6, ^{15}N_3]$-L-histidine (Cambridge Isotope Laboratories, Andover, MA), the labelled histidine was included together with 19 other (unlabeled) amino acids in the growth medium as described by Raap et al. (1990).

Isolation of the LH2 complex

The LH2 complex of *Rps. acidophila* strain 10050 was prepared as described elsewhere (Cogdell and

Type 1

Type 2
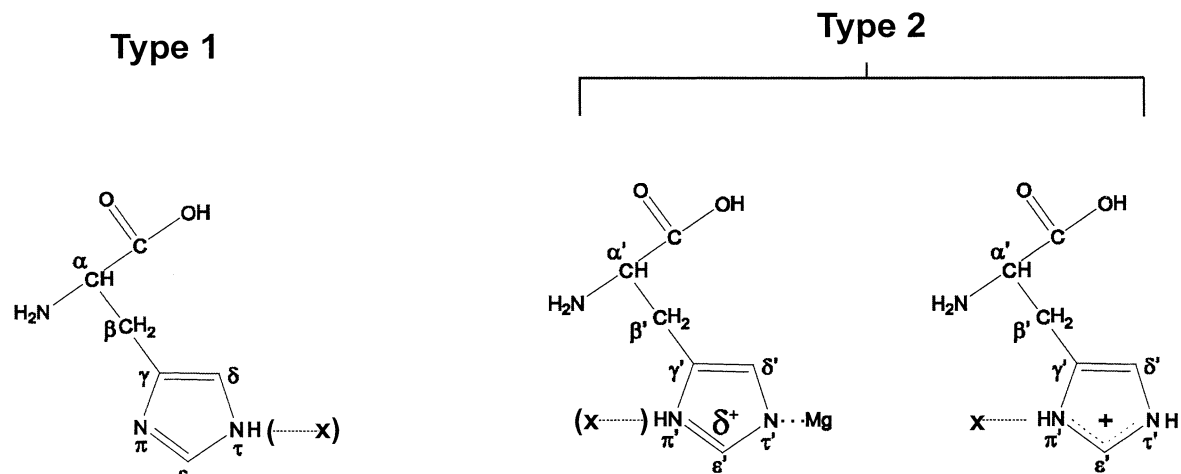
Scheme 1.

Hawthorntwaithe, 1993). In brief, chromatophores were prepared and incubated for 2 h in a 2% LDAO solution at 4 °C and subsequently ultracentrifuged overnight using a discontinuous sucrose gradient. The purified LH2 was dialysed against 30 mM Tris/EDTA buffer (pH 8.0) containing 0.3% LDAO for 24 h and concentrated to an OD_{800} of 330 cm^{-1} using a Filtron 30-kDa filter.

The ^{13}C and ^{15}N-isotope enrichment of the histidine residues in LH2 was measured by gas chromatography and electron impact mass spectrometry (GC-MS) as described earlier (Alia et al., 2001). Incorporation of [^{13}C$_6$,^{15}N$_3$]-L-histidine in the LH2 complex was more than 95%. Detergent-solubilized LH2 was used throughout all measurements presented in this paper. Before and after taking the CP/MAS NMR data on the LH2 preparations, the electronic absorption spectra were measured with a Shimadzu UV-160 A spectrometer. No change in the absorption spectrum was detected, which confirms that the integrity and quality of the sample were maintained during the MAS NMR measurement procedure. For low pH experiments, the pH of the sample was adjusted to 4.0 by adding small aliquots of 1 M HCl while monitoring the pH with a microelectrode.

MAS NMR measurements

For CP/MAS NMR experiments, 0.1 ml of an LH2 sample with an OD_{800} of 330 cm^{-1} was loaded into a 4 mm MAS rotor. The solid-state CP/MAS spectra were recorded with a DMX-750 NMR spectrometer equipped with a 4 mm double resonance CP/MAS probe (Bruker, Karlsruhe, Germany) operating at 750 MHz for ^{1}H and 188 MHz for ^{13}C. The experiments were performed at T = 220 K with a spinning rate of 12 kHz. The pulse sequence for the 2-D heteronuclear (^{1}H-^{13}C) dipolar correlation (HETCOR) spectra is shown in Figure 2. The protons evolve during t_1 under suppression of the ^{1}H homonuclear dipolar interactions with the phase-modulated Lee-Goldburg sequence (PMLG-3) (Vinogradov et al., 1999, 2000). The PMLG irradiation strength was 74.4 kHz. Heteronuclear polarisation transfer was achieved during a short CP mixing time of 256 μs, with a ramped CP spin-lock to broaden the CP matching profile at high MAS rates. The ^{13}C free induction decay (FIDs) were recorded in the t_2 domain, using proton decoupling with the two-pulse phase modulation (TPPM) scheme (Bennet et al., 1995).

Computation of ring current shifts on histidine in LH2

The electronic structure and ring current induced shifts for Mg coordinating histidines in LH2 were calculated using Density Functional Theory (DFT) calculations. The DFT calculations were performed by using Gaussian 98 (Gaussian, Carnegie, PA) on an IBM SP2 scalable computer. The crystal structure of LH2 of *Rps. acidophila* strain 10050 (McDermott et al., 1995), was acquired from the RCSB Protein Data Bank. From this structure one of the coordinating B850 BChl *a*/His complexes was taken. The histidine molecule was removed while providing the Hδ and Hε coordinates for the calculations. To the BChl *a* molecule H atoms were attached since they are not given by the crystal structure. The phytyl tail was truncated with a methyl group. First, the C-H bonds were optim-
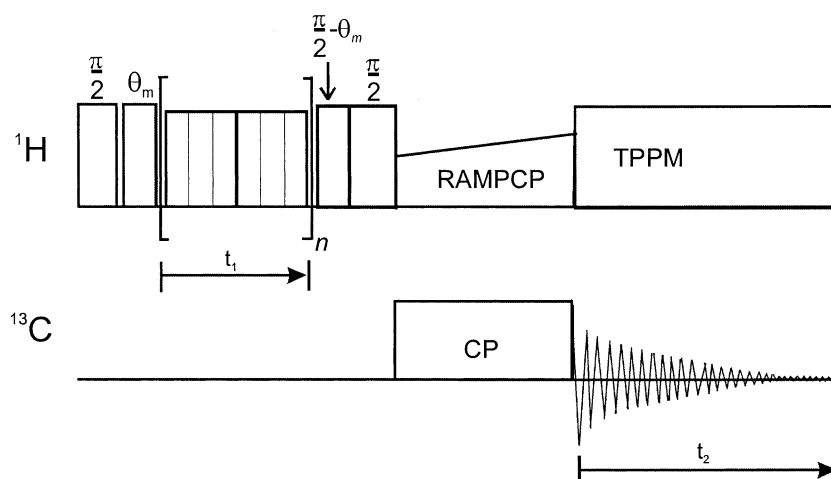
Figure 2. Pulse program used for 2-D PMLG-decoupled ^{1}H-^{13}C heteronuclear dipolar correlation spectroscopy. The PMLG sequence in t_1 consists of series of 6 pulses with well-defined phases of 34.6°, 103.9°, 173.2°, 353.2°, 283.9 and 214.6° respectively. The first t_1 increment is 20 μs and subsequently 128 steps of 40 μs each. $\pi/2 - \theta_m = 35.3°$.

ised at the semi-empirical PM3 level. The electronic structure was optimised at BLYP/6-31G(d,p) level. Subsequently, Mulliken charges and NICS (nucleus independent chemical shift) values were calculated. The NICS values were calculated for ghost atoms located at the Hδ and Hε sites. NICS values were also calculated by using standard C-H bond lengths of 0.1 nm without further optimisation, which yielded similar results as with optimised C-H bond lengths.

Results and discussion

^{1}H assignment of histidine residues in LH2

Figure 3 shows 2D ^{1}H-^{13}C dipolar correlation spectra of LH2 recorded with the pulse sequence depicted in Figure 2 with a cross polarisation mixing time of 256 μs. ^{1}H resonances of the Type 1 and Type 2 histidines (Scheme 1) (Alia et al., 2001) can be readily assigned through correlations with ^{13}C that show unique carbon shifts. The resonances of the δ- and ε−^{1}H of the imidazole ring of Type 1 histidine (β-His 12 and α-His 37) appear at 6.2 and 7.4 ppm, respectively (Figure 3 and Table 1). Interestingly, ^{1}H resonances of the imidazole ring of the Type 2 histidine are observed at two different positions. The δ-^{1}H resonate with 6.5 and 2.6 ppm while ε-^{1}H responses appear at 7.9 and at 4.2 ppm (Figure 3 and Table 1). In our previous studies using 2D homonuclear (^{13}C-^{13}C) dipolar correlation data and ^{15}N-^{13}C double CP/MAS NMR experiments, Type 2 histidines were assigned to

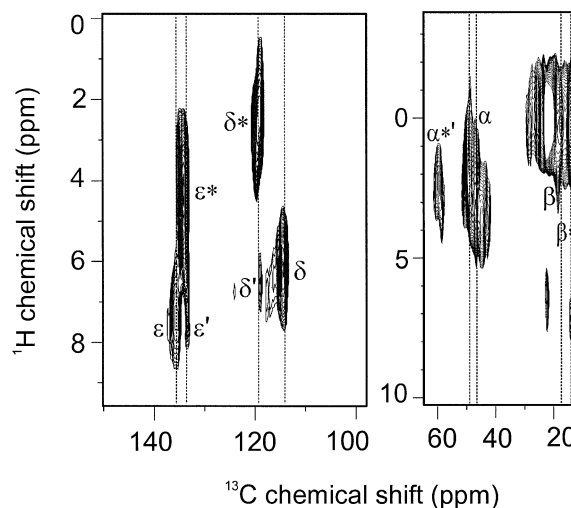*Figure 3.* 2-D heteronuclear (^{1}H-^{13}C) dipolar correlation spectrum of [^{13}C$_6$,^{15}N$_3$]-histidine labeled LH2 complex collected in a field of 17.6 T. The spectrum was recorded with a spinning frequency $\omega_r/2\pi = 12$ kHz at a temperature of 230 K and a CP contact time of 256 μs. The ^{1}H homonuclear interactions were suppressed with PMLG irradiation during proton evolution, applying an RF power corresponding with a nutation frequency of 74.4 kHz. The spectrum is divided into two parts that are plotted with a different range of contour levels to improve the representation of the data. Cross peaks from the cationic histidines (Type 2A) are indicated by (') and cross peaks from the histidines bound with B850 (Type 2B) are indicated by (*).

those which are either bound to the central magnesium atom of B850 (β-His30 and α-His31) or fully protonated histidines (β-His41). The ^{1}H resonance of δ- and ε- at 6.5 and 7.9 respectively, in Figure 3, from Type 2

Table 1. [1]H chemical shift assignment of histidines in the LH2 complex from *Rps. acidophila*

Protons type	Chemical shifts (ppm)			
	Type 1 His (neutral)	Type 2A His (cationic)	Type 2B His (Mg bound)	Ring current shifts[a] (RCS)
Hα	3.00	3.90	2.60	0.40
Hβ	0.30	0.14	0.28	0.02
Hδ	6.20	6.50	2.70	3.50
Hε	7.90	7.90	4.20	3.70

[a](RCS = Type 2B His – Type1 His).

Table 2. Comparison of solid state ^{13}C chemical shifts of the imidazole ring of histidine in the neutral and cationic state in model systems with those of histidines in LH2 complex from *Rps. acidophila.* All ^{13}C data are relative to the chemical shift of TMS

Carbon type	Chemical shifts (ppm)		
	Type 1 His (neutral)	Type 2A His (cationic)	Type 2B His (Mg bound)[a]
Cγ	133.7	125.1	125.1
Cδ	113.5	118.3	121.9
Cε	136.5	133.5	136.6

[a]Renormalised ^{13}C shifts.

Figure 4. 2-D heteronuclear ([1]H-^{13}C) dipolar correlation spectrum of [[13]C$_6$,[15]N$_3$]-histidine labeled LH2 complex at pH 4.0. The NMR experimental conditions were same as mentioned in the legend for Figure 3.

histidines could be easily assigned to β-His41, which carries a net positive charge (Type 2A). The ^{1}H resonances of δ- at 2.6 and ε- at 4.2 ppm can be assigned to histidines (β-His30 and α-His31) which are in close proximity to B850 (Type 2B). The upfield shift of δ- and ε-^{1}H for the Mg bound His relative to the other histidines in LH2, reflects the ring current shift effect experienced by nuclei in close proximity to the conjugated porphyrin ring system of B850 that could not be resolved in the ^{13}C data reported previously (Alia et al., 2001).

According to the X-ray structure of LH2 from *Rps. acidophila*, β-His30 is located above the aromatic ring of B850, with the δ and ε carbons at a distance of approximately 3.38 Å and 3.31 Å, respectively (Mc-Dermott et al., 1995). Similarly α-His31 is located above B850, with its δ and ε carbons at a distance of approximately 3.54 Å and 3.12 Å, respectively (Figure 1). In the present study, ring current induced shifts for Mg coordinating histidines in LH2 were calculated using Density Functional Theory (DFT) calculations. From the computation of ring-current shifts for the δ and ε hydrogens of histidine due to the B850 in
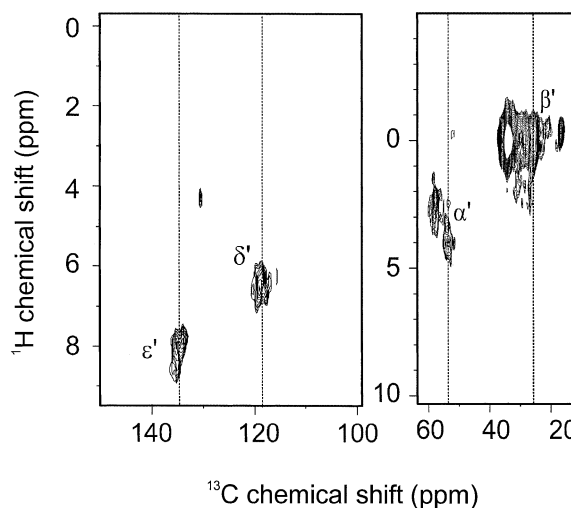
LH2 yields estimates of approximately −3 ppm and −4 ppm for the δ and ε protons of β-His30 and α-His31, respectively. The MAS NMR data show ring current shifts of −3.5 for the δ and −3.7 ppm for the ε protons of β-His30 and α-His31, well in line with the theoretical estimates. The ^{1}H results thus clearly demonstrate that the histidines in close proximity of B850 (β-His30 and α-His31) experience a ring current. This is due to the extensive delocalisation of electrons in the porphyrin ring of B850. These results also suggest that supermolecular π interaction in the assembly of 18 B850 ring in LH2 does not significantly affect the ground state electronic structure of individual BChl *a*/His complex.

To validate the assignment of ^{1}H resonances from neutral (β-His12 and α-His37), fully protonated (β-His41) and magnesium bound histidines (β-His30 and

α-His31) in LH2, 2D ^{1}H-^{13}C dipolar correlation spectra of LH2 at pH 4.0 have been measured. As shown in Figure 4, δ- and ε-^{1}H of all 5 histidines in LH2 appeared at around 6.48 and 7.9 ppm respectively. At pH 4.0 all histidines are fully protonated and also the N...Mg interaction of β-His30 and α-His31 with B850 breaks at this pH (Alia et al., 2001). This confirms that the ^{1}H responses at 6.48 and 7.9 ppm from δ- and ε- positions in Figure 3 correspond with β-His41, which carries a net positive charge at physiological pH in LH2. The intensity of the ^{1}H-^{13}C cross peaks of ε and δ in Figure 3 also indicate that out of five histidines in LH2, one histidine carries full positive charge (δ and ε at 6.5 and 7.9 ppm), two of the histidines are neutral (δ and ε at 6.2 and 7.4 ppm), while the other two are coordinated to the Mg^{2+} of B850 with partial positive charge character (δ and ε at 2.6 and 4.2 ppm).

^{13}C assignment of histidine residues in LH2

On the basis of the ^{1}H-^{13}C cross peaks in 2D ^{1}H-^{13}C dipolar correlation spectra, an unambiguous assignment of ^{13}C chemical shifts of δ- and ε- carbons of the imidazole of the magnesium bound histidines (β-His30, α-His31) has been made in the current paper, which has not been feasible in the previous study (Alia et al., 2001). The resonances from δ- and ε- carbons of Mg bound histidines appeared at 118.3 and at 133.5, respectively (Figure 3). Although the shifts for the δ- and ε-carbons are similar to the shifts observed for the cationic histidine (β-His 41) on the carbon scale, in the ^{1}H dimension a clear separation between cationic histidines and the Mg bound histidines (Figure 3) is observed.

In principle the ring current shifts on the ^{13}C nucleus should be comparable to the shifts experienced by the directly bounded ^{1}H nuclei (Blanchard et al., 1997). Since δ- and ε-protons experience a ring current shift of ∼3.6 ppm, we use this information to renormalise the ^{13}C chemical shift of δ- and ε-carbons by subtracting the ring current effect. The renormalised shifts for the ε-carbons of the Mg-bound histidines are 136 ppm, similar to the ε-carbon of the neutral histidines. On the other hand, the renormalised value for the δ-carbon of Mg-bound histidines is 121.9 ppm. This value is different from the shift for the δ-carbon of either neutral or fully positively charged histidine. These results confirm that Mg bound histidines have a different electronic structure from either fully positively charged or fully neutral histidine species. The renormalised ^{13}C shifts can be combined with the in-
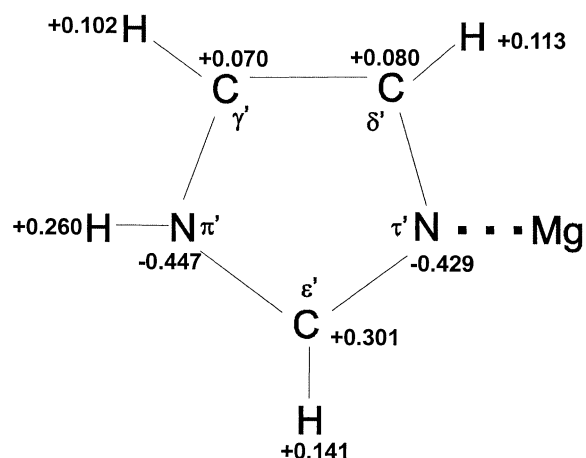

Figure 5. Net Mulliken charge distribution on H, C and N atoms in histidines coordinating with Mg.

formation deduced from the anisotropic shift tensor of Mg bound nitrogen, providing converging evidence for a BChla$^{δ-}$/His$^{δ+}$ complex in LH2.

Finally, the electronic structure of a B850-ligating histidine has been calculated using density functional theory calculations. The net Mulliken charge distribution on H, C and N atoms in the imidazole ring of Mg coordinating histidine derived from DFT calculations is represented in Figure 5. The calculated Mulliken charges indicate a partial positive charge of +0.19 on imidazole ring of Mg-coordinating histidines. These results thus confirm an important conclusion from our previous paper that the coordinating histidines are partially positively charged in a neutral BChl *a*/His complex, which is capable of buffering a significant amount of positive charge (Alia et al., 2001).

In summary, we have shown that the electronic structure of Mg bound histidines can be clearly distinguished from other hsitidines in supermolecular assembly of LH2 complex using two-dimensional heteronuclear (^{1}H-^{13}C) dipolar correlation MAS NMR spectroscopy. This leads to a refined assessment of the electronic structure of B850/His complex in LH2. Such study would be invaluable to probe the magnesium-histidine interactions in several other photosynthetic pigment protein complexes including reaction centres.

Acknowledgements

164

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