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Bacteriochlorophyll/imidazole and chlorophyll/imidazole complexes are negatively charged in an apolar environment

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Abstract

600 MHz ^{15}N -NMR and 2D homonuclear ^1H - ^1H and heteronuclear ^1H - ^{15}N -NMR data for bacteriochlorophyll *a*/imidazole and chlorophyll *a*/imidazole have been recorded. Unambiguous assignments of the ^{15}N signals of the two nitrogens of imidazole ligated to the Mg of the bacteriochlorophyll *a* or chlorophyll *a* were obtained. It follows that the imidazole in complex is deprotonated, which implies that the chlorophyll/imidazole carries a full negative charge in the apolar solvent environment. This information is potentially useful in characterizing the nature of the magnesium–histidine interaction and the charge state of chlorophylls coordinated by histidine in photosynthetic pigment protein complexes. © 2000 Elsevier Science B.V. All rights reserved.

1. Introduction

Interaction of histidine with magnesium has been suggested in all bacteriochlorophyll-protein complexes with known structures [1–3]. The spectroscopic evidence for this interaction is mainly based on resonance Raman studies on bacterial antenna complexes which show that histidine residues in the apoprotein are liganded to Mg at the centre of the bacteriochlorophyll *a* (BChl *a*). These studies are confirmed by in vitro Raman experiments on BChl *a* in the presence of substituted imidazole [4].

Recently, the first MAS NMR results on the ground state electronic structure of histidine imidazole side chain nitrogens were obtained from bacterial reaction centers that were labelled with τ - ^{15}N or π - ^{15}N histidine [5]. These results were surprising, since evidence was found that BChl *a*/histidine complexes in the reaction centres are carrying a spinless charge. However, the assignment of ^{15}N chemical shifts of histidines in pigment protein complexes such as reaction centres of photosynthetic bacteria (BRC) and plants (PSII) is difficult. In particular, the ^{15}N chemical shifts for imidazole nitrogens of histidines coordinating with magnesium of chlorophylls are not known.

To provide a solid basis for the membrane protein complex studies, a model compound study is performed. The assignment of the magnesium–imidazole ^{15}N -NMR resonance in the chlorophyll *a* (Chl *a*)/imidazole and the BChl *a*/imidazole

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complex in THF is obtained. The surprising result of the studies is that due to deprotonation of the imidazole, both the Chl *a*/imidazole and BChl *a*/imidazole complexes are negatively charged.

2. Materials and methods

2.1. Synthesis of [$^{15}\text{N}_2$] imidazole

The procedure of Gridnev and Mihaltseva [6] was followed for synthesizing [$^{15}\text{N}_2$]imidazole with slight modifications. In brief, 12.2 ml of 40% aqueous solution of glyoxal, 8.1 ml of 37% aqueous solution of formaldehyde, 11.1 g $^{15}\text{NH}_4\text{Cl}$ and 10 drops of 35% HCl were mixed for 90 min at 95°C. The mixture was first cooled and subsequently KOH was added slowly (in ca. 15 min) dropwise until a pH = 14 was reached. The final reaction mixture was evaporated to dryness at 40–50°C by using a rotary evaporator. The brown solid product was scraped off the wall of the flask and transferred to a vacuum sublimation apparatus where the crude [$^{15}\text{N}_2$]imidazole sublimed away readily at ca. 125°C under vacuum. The crude [$^{15}\text{N}_2$]imidazole was recrystallized from toluene which yielded highly pure white, crystalline [$^{15}\text{N}_2$]imidazole. The yield was ~53% based upon the starting ammonium salt.

2.2. Synthesis of [$^{15}\text{N}_2$] 1-methylimidazole

0.5 g [$^{15}\text{N}_2$]imidazole in 30 ml anhydrous THF was added under inert conditions to a 100 ml, completely dried three-necked flask, previously flushed with argon, and equipped with a mechanical stirrer, an additional funnel, and a condenser. 4.6 ml of 1.6 M butyl lithium was added to the flask with the help of a syringe under inert conditions. To the resulting mixture, 1 ml of methyl iodide was added dropwise and the mixture was refluxed for 3 h at 70 °C. The reaction mixture was then transferred to a 250 ml round bottom flask and the solvent was evaporated by using a rotary evaporator at 40–50°C. The resulting residue was distilled twice which yielded highly pure [$^{15}\text{N}_2$] 1-methylimidazole as a colorless liquid. The yield was ~25%-based upon the starting [$^{15}\text{N}_2$]imid-

azole. The identity and purity of the product were confirmed by ^1H -NMR by comparison with an authentic sample of 1-methylimidazole.

2.3. Isolation of pigments

BChl *a* and bacteriopheophytin *a* (BPheo *a*) were extracted from cells of *Rhodobacter sphaeroides* (R26) and purified by normal phase HPLC as described previously [7]. Chl *a* and Pheophytin *a* (Pheo *a*) were extracted from spinach and purified using the method described in [8].

2.4. Complex formation

For the preparation of complexes of imidazole or 1-methyl imidazole with BChl *a*, BPheo *a*, Chl *a* or Pheo *a* pigments, the doubly enriched imidazole or 1-methyl imidazole was co-dissolved with one of the pigments as described by Robert [9]. Imidazole concentrations were between 3 and 300 mM in tetrahydrofuran (10% d8) at 25°C. A pigment concentration of 4 mM was found to be optimal and was used for all experiments.

To obtain the imidazole anion, imidazole (80 mM) was reacted with equimolar concentration of tetrabutylammonium hydroxide-30 hydrate ($\text{C}_{16}\text{H}_{37}\text{NO} \cdot 30\text{H}_2\text{O}$). The resulting mixture was dissolved in THF (10% d8) and the water was removed by using molecular sieves (3A, 8–10 MESH; Acros Organics, New Jersey, USA). The complex of anion imidazole with BChl *a* was made by titrating the anion imidazole with BChl *a* in THF.

2.5. NMR measurements

All NMR spectra were recorded at 25°C with a Bruker 600 MHz DMX NMR spectrometer equipped with a pulsed field gradient accessory (Bruker, Karlsruhe, Germany). 2D homonuclear ^1H – ^1H and heteronuclear ^1H – ^{15}N experiments were performed with a 5 mm inverse triple high resolution probe with an actively shielded z -gradient coil. For 1D ^{15}N experiments a 5 mm broadband high resolution probe was used ^{15}N chemical shifts are referenced using an external standard of $^{15}\text{NH}_4^{15}\text{NO}_3$ saturated in water at 22.3 ppm in reference to liquid ammonia. ^1H chemical

shifts were calibrated using the THF signal at 1.73 ppm downfield from tetramethylsilane (TMS) as an internal standard.

The ^1H – ^{15}N correlations were determined by measuring heteronuclear multiple-bond correlation (HMBC) [10] spectra at 25°C. Two dimensional gradient selected ^1H – ^1H correlation spectroscopy (COSY) [11] spectra of the complex were recorded with a relaxation delay of 1 s. The NMR spectra were processed with X-WINNMR (Bruker) on Silicon Graphic Indy workstations.

3. Results and discussion

The 600 MHz ^{15}N -NMR spectrum of the BChl *a*/imidazole complex in THF at 25°C is shown in

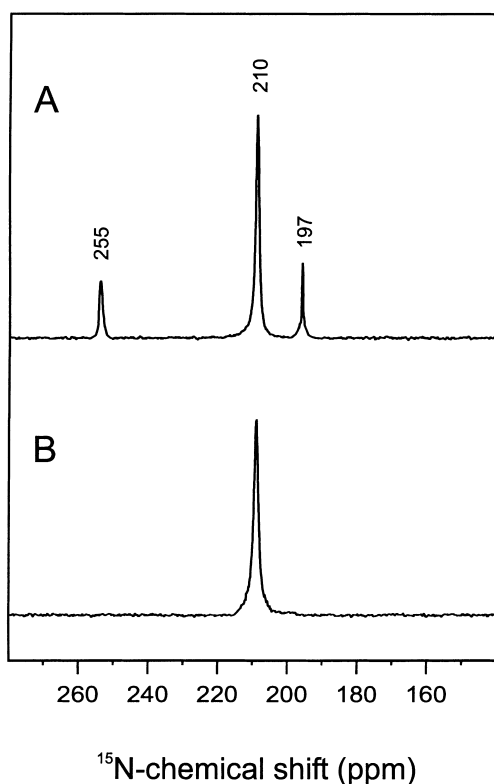
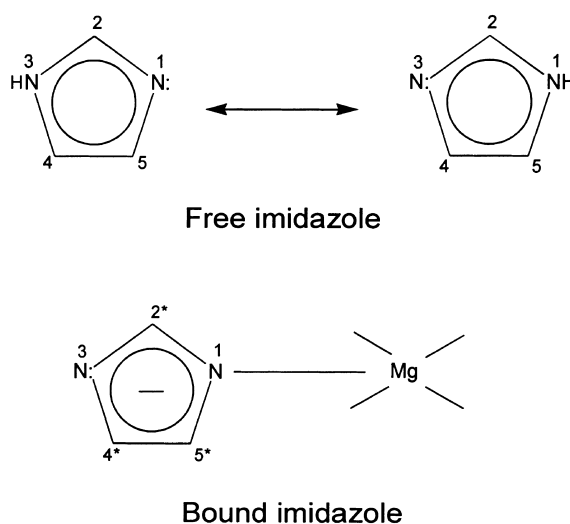


Fig. 1. The 600 MHz one-dimensional ^{15}N -NMR spectrum of imidazole (enriched to 95% ^{15}N at both nitrogens) complexed with BChl *a* (A) or BPheo (B) in THF (10% d_8) at 25°C. For the ^{15}N -NMR spectrum 5000 scans with 0.3 s repetition time were measured. The numbering system of the imidazole ring is indicated in Scheme 1.

Fig. 1a (Scheme 1). The signal from free imidazole is observed as a single resonance at 210 ppm due to the tautomerization reaction, which is very fast at ambient temperature [12]. Two additional resonances are observed at 255 and 197 ppm, respectively.

The response at 255 ppm is most easily assigned to a lone pair imidazole ($>\text{N}:$). This is in agreement with the $>\text{N}:$ shifts reported in the literature [13]. In that case, the signal at 197 ppm should be assigned to the other nitrogen of imidazole coordinating the magnesium of BChl *a* ($>\text{N}-\text{Mg}$). A reversed assignment is unlikely, since the signal at 197 ppm is well outside the range expected for protonated imidazole nitrogen ($>\text{NH}$). The range for imidazole nitrogens is between 177 ppm for $>\text{NH}$ and 186 ppm, when the proton participates in a strong hydrogen bond ($>\text{NH}-\text{X}$) [13]. As shown in Fig. 1b, co-dissolving imidazole with BPheo *a* only yields the response at 210 ppm from the imidazole in tautomeric equilibrium. Since BPheo *a* does not contain an Mg^{2+} ion in its center, the data in Fig. 1b suggest that Mg is important for ligation of imidazole with BChl *a*. Hence, the resonance 197 and 255 ppm, respectively, arise solely from the bound imidazole. These results also confirm that the chemical shift at 197 ppm arise from nitrogen coordinated with magnesium.



Scheme 1.

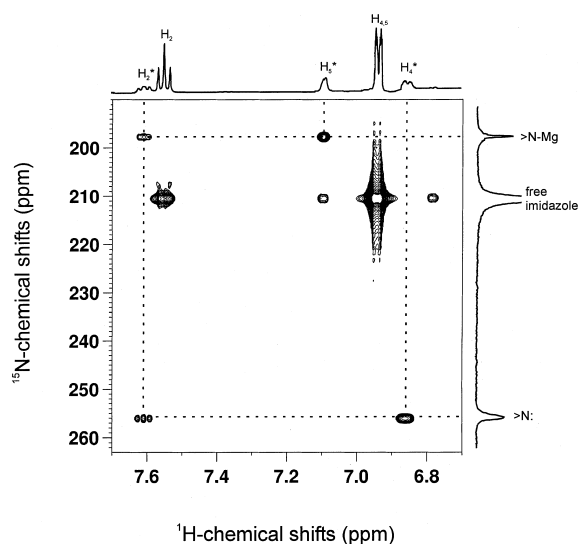


Fig. 2. 600 MHz ^{15}N - ^1H HMBC spectrum of imidazole (enriched to 95% ^{15}N at both nitrogens) complexed with BChl *a* in THF (10% d_8) at 25°C , showing the cross-peaks between proton and nitrogens of bound imidazole. * resonance of ^1H in the imidazole ring bound with BChl *a*.

To further characterize the ^{15}N resonance of bound imidazole, a 2D HMBC data set was measured. Fig. 2 shows the ^{15}N - ^1H HMBC spectrum of BChl *a*/imidazole in THF solution. The trace on top of the dataset shows the ^1H spectrum from the BChl *a*/imidazole complex.

The resonances from the bound imidazole ring are clearly seen and are designated H_2^* , H_4^* and H_5^* . The strong resonances from the free imidazole are indicated as H_2 , H_4 and H_5 . The assignments were confirmed by a standard ^1H - ^1H COSY experiment (data not shown). The H_2^* , H_4^* and H_5^* signals of bound imidazole were used for the assignment of two ^{15}N signals of bound imidazole in the HMBC spectrum. The ^{15}N chemical shift of free rapidly tautomerizing imidazole at 210 ppm exhibits strong correlations with H_2 and $\text{H}_{4,5}$. For the bound imidazole, the ^{15}N signal at 255 ppm exhibits correlations with the H_2^* and H_4^* signals and therefore it is assigned to N_3 of bound imidazole. The ^{15}N signal at 197 ppm exhibits a correlation with H_2^* and H_5^* and it is assigned to N_1 which is coordinated to the Mg of the BChl *a*. No ^{15}N - ^1H correlation could be obtained for any bound imidazole with BPheo *a* (data not shown). Thus, the ^{15}N - ^1H correlation spectra led us to an unequivocal assignment of the two nitrogens of imidazole in complex with BChl *a*.

The same set of experiments was repeated with the complex of plant Chl *a* in complex with imidazole. The data are summarized in Table 1. The ^{15}N -NMR spectrum of plant Chl-imidazole complex also shows two distinct peaks from the bound imidazole, at 198 and 257 ppm, respectively. These chemical shifts are almost the same as the chemical

Table 1

^{15}N Chemical shifts of imidazole and its derivatives complexed with BChl *a* and Chl *a*

Compound	Nitrogen type		^{15}N chemical shifts (ppm)
Imidazole	Tautomer		210
Imidazole-BChl <i>a</i> complex	N1	>N-Mg	197
	N3	>N:	255
Imidazole-Chl <i>a</i> complex	N1	>N-Mg	198
	N3	>N:	257
Anionic imidazole	N1	>N:	249
	N3	>N:	249
Anionic imidazole ^a -BChl <i>a</i> complex	N1	>N-Mg	196
	N3	>N:	255
1-Methylimidazole	N1	>N-CH ₃	156
	N3	>N:	257
1-Methylimidazole + BChl <i>a</i>	N1	>N-CH ₃	156
	N3	>N:	257

^a Formed by reacting imidazole with an equimolar concentration of tetrabutylammonium hydroxide.

shifts obtained with the BChl *a*/imidazole complex. The resonances at 198 and 257 ppm were not observed in a Pheo *a*/imidazole mixture (data not shown). An unambiguous assignment of N₁ and N₃ was again made on the basis of cross-peaks of these nitrogens with H₂ and H₄ or H₅ in 2D ¹⁵N–¹H-HMBC spectra of the Chl *a*/imidazole complex. Resonances at 257 and 198 ppm were similarly assigned to N₃ containing a lone pair of electron (>N:) and N₁–Mg in Chl *a*, respectively.

These results show that the chemical shift difference between imidazole nitrogens which bear a hydrogen (>NH) [13] and those which bear no hydrogen and coordinating with magnesium (>N–Mg) is about 30 ppm. Our model studies put a question mark on the assignment inferred by Zysmilich and McDermott [14]. These authors suggest that for bacterial reaction centres, the chemical shift difference between the imidazole nitrogen responses of histidines coordinating with bacteriochlorophyll and a protonated N–H is ~58 ppm.

In a control experiment, the one nitrogen of imidazole was blocked with a methyl group in methylimidazole. We have co-dissolved [¹⁵N₂]-1-methylimidazole and BChl *a*. No complex could be formed between the free nitrogen of 1-methylimidazole and the magnesium of BChl *a* (Table 1). In another set of control experiments, a stable complex of imidazole with BChl *a* was formed in two steps. First an anionic imidazole was prepared by transferring the proton of the imidazole nitrogen to a tetrabutylammonium hydroxide counterion. Subsequently, the anion imidazole was titrated with BChl *a* in THF. The ¹⁵N-NMR spectra of the resulting complex showed again ¹⁵N chemical shifts of 197 and 255 ppm, corresponding with nitrogens of imidazole coordinating the magnesium of BChl *a* (>N–Mg) and imidazole nitrogen not bearing a hydrogen (>N:), respectively (Table 1). It is not unlikely that in case of normal BChl *a*/imidazole complex the proton of the imidazole is transferred to free imidazole pool.

Our results provide an assignment of the magnesium-imidazole ¹⁵N-NMR resonance in Chl *a*/imidazole and BChl *a*/imidazole complex. This can serve as a useful model for identifying the nature

of the magnesium-histidine interaction in many photosynthetic pigment protein complexes. From our results, it appears that when the imidazole nitrogen is bound to Mg, the other nitrogen is deprotonated, and the complex carries a net negative charge. In a way, the Mg²⁺ can replace the role of the proton in a free and neutral imidazole ring. Mg is the smallest ion that can be put into the macroaromatic porphyrin-type system, which may explain why it is possible to generate such a complex of imidazole and chlorophyll in an apolar environment. This is interesting, considering the importance of the chlorophyll-imidazole structural motif in light harvesting and charge separation in photosynthesis.

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