Paramagnetic Inversion of the Sign of the Interference Contribution to the Transverse Relaxation of the Imido Protons of the Coordinated Imidazoles in the Uniformly ^{15}N Labeled Cytochrome c_3

Andre Kim, In-Chul Jeong, Yoon-Bo Shim, Shin-Won Kang, and Jang-Su Park*

Department of Chemistry and Chemistry Institute for Functional Materials, Pusan National University, Pusan 609-735, Korea
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In the spectrum of uniformly ^{15}N labeled cytochrome c_3, the relative linewidths of the doublet peaks of the ^{15}N-coupled imido proton of the coordinated imidazole group were reversed on oxidation. This inversion was explained by the interference relaxation process between the electron-proton dipolar and ^{15}N-^{1}H dipolar interactions. The inversion can be used to assign the imido protons of the coordinated imidazole groups in heme proteins.

Keywords : *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF), Imido proton, HMQC, ^{15}N-Labeled cytochrome c_3.

Introduction

It has been indicated that the relaxation mechanism of two coupled unlike half spins involve the interference cross correlation relaxation, leading to the different linewidths of the doublet peaks. Such phenomena were reported for the NH proton signals of tRNA and ^{19}F and ^{31}P signals of pyridoxal fluorophosphate in glycogen phosphrylase b.[1,2,3] Theoretical analysis of such relaxation mechanism has been carried out as well.[4,5] Taking advantage of the interference term, the chemical shift anisotropy can be estimated even in the solution NMR. A set of pulse sequences have been developed to measure the relaxation interference effect and to estimate the reduced chemical shift anisotropies of the amide nitrogen-15 of a protein.[6] The obtained ^{15}N chemical shift anisotropy of the amide in ubiquitin was correlated to the order parameter of the NH bond at each position, suggesting that it can be used for the analysis of the dynamic structure of a protein.[6] The analysis of the chemical shift anisotropy of the amide protons for three proteins has been reported quite recently.[7,8,9]

In the spectrum of uniformly ^{15}N labeled cytochrorme c_3, we have found the inversion of the relative linewidths of the doublet peaks of the ^{15}N-coupled imido proton of the coordinated imidazole group on oxidation. Cytochrome c_3 is a tetraheme protein of a sulfate-reducing bacterium. All the hemes take the low spin state. The heme irons are diamagnetic and paramagnetic in the fully reduced and fully oxidized states, respectively. The inversion of the relative linewidths was elucidated by the relaxation interference effect induced by the paramagnetic center in this present work.

Experimental Section

Sample preparation. Uniformly ^{15}N-labeled protein was obtained from *Desulfovibrio vulgaris* Miyazaki F grown in Medium C containing 0.3 g/L 99% $^{15}NH_4Cl$ (Isotech, Inc.) as sole nitrogen source.[10] For NMR experiments, a trace amount of hydrogenase was added to a 2 mM cytochrome c_3 solution (molar ratio, *ca.* 0.001) in 30 mM phosphate buffer (p^2H 7.0). The hydrogenase was purified from *Dv*MF cells according to the reported method.[11] Full reduction of cytochrome c_3 was achieved by controlling the ratio of hydrogen and argon gassed in an NMR tube. The samples for heteronuclear NMR typically compromised 4 mM protein in 90% H_2O/10% D_2O, containing 30 mM phosphate buffer, pH 5.0 and 7.0 for oxidized and reduced state, respectively. The 2D ^{1}H-^{1}H NMR experiments were performed with 8 mM sample dissolved in 90% H_2O/10% D_2O or pure D_2O 30 mM phosphate buffer, pH 5.0 and 7.0 for oxidized and reduced state, respectively.

NMR spectroscopy. HMQC (heteronuclear multiple quantum correlation spectroscopy) were performed on a Varian UNITY INOVA-500 spectrometer at 30 °C. Matrix size was 4 K × 512, and spectral widths were 10 × 7.6 kHz and 13.2 × 7.6 kHz for the reduced and oxidized states, respectively. The curve fitting was carried out for the spectra with the digital resolution of 1.2 Hz/point and 1.6 Hz/point for the reduced and oxidized states, respectively. The linewidths were obtained from the slices of 2D HMQC spectra because the NH signals were overlapped with many other proton signals in the spectrum of the reduced cytochrome c_3. The linewidths were obtained from the slices of 2D HMQC spectra because the NH signals were overlapped with many other proton signals in the spectrum of the reduced cytochrome c_3. The linewidths were obtained by curve fitting assuming a lorentzian as a line-shape.

All ^{1}H 2D-NMR spectra were recorded at 30 °C on a Varian UNITY INOVA-500 NMR spectrometer at 30 °C. The NOESY (nuclear Overhauser effect spectroscopy) in the reduced state was measured with a mixing time 100 ms, spectral width 8680 Hz in both dimensions. The digital resolutions were 8.48 and 4.24 Hz/point for F1 and F2, respectively. The NOESY in the oxidized state was measured with a mixing time 25 ms, spectral width 35970 Hz in both dimensions.

*To whom correspondence should be addressed. Tel: +82-51-510-2294, Fax: +82-51-516-7421; e-mail: jaspark@hyowon.cc.pusan.ac.kr

The digital resolutions were 35.13 and 17.56 Hz/point for F1 and F2, respectively.

Results and Discussion

The assignment of the NMR signals of the proton and nitrogen-15 of the polypeptide backbone has been carried out both for the fully reduced and fully oxidized states. During the assignment, it has turned out that the change in the oxidation state reverses the relative linewidths of eight ^{15}N-coupled proton signals. One of those is presented in Fig. 1. While the linewidth of the signal at lower field is broader than that at higher field in the fully reduced state, the linewidth at higher field becomes broader than that at lower field in the fully oxidized state. The former is the case with all ^{15}N-coupled protons in the fully reduced state. The assignment of the signals shown in Figure 1 have been established by the sequential assignment of the backbone protons and ^{15}Ns and assignment of the side chain protons. The sections of the 2D NOESY spectra including the signals of interest are presented in Figure 2, indicating that they are clearly the imido proton of histidine.

On the basis of these results, the signals in Figure 1 were attributed to the imido proton of His 52 coordinated to heme 2 of cytochrome c_3. The detail of the sequential assignment

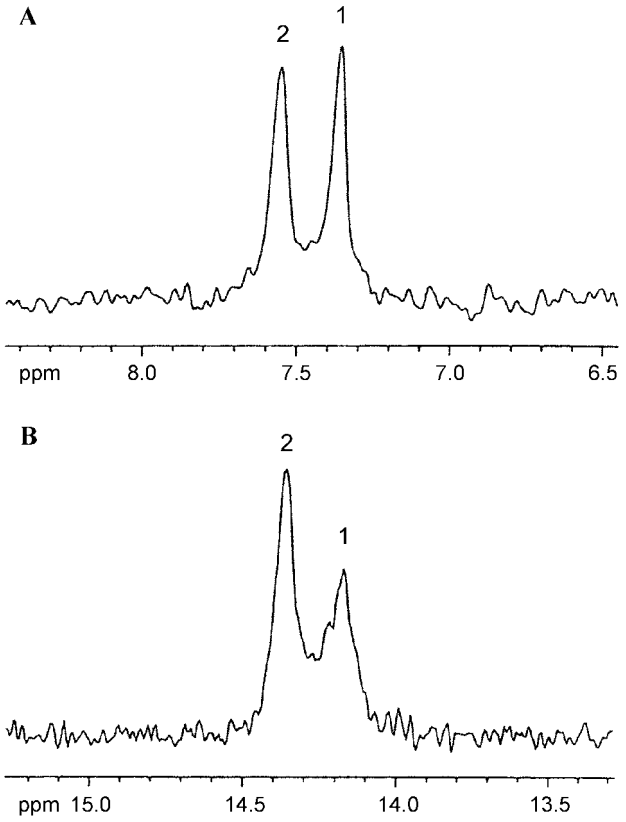

Figure 1. Proton NMR spctra of the imidazole imido protein of His 52 of uniformly ^{15}N labeled cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki F in the fully reduced (A) and fully oxidized (B) states. The label on the top of signals strand for the ^{15}N-coupled components shown in Eqs. (1) and (2).

will be published elsewhere. The assignment indicates that the inversion of the relative linewidths of the doublet peaks of a coupled proton on oxidation should be induced by the appearance of the paramagnetic center. According to Goldman, the transverse relaxation rates of the two resonance lines of a proton coupled with ^{15}N ($\omega_H^{(1)} = \omega_H + (1/2)J$ and $\omega_H^{(2)} = \omega_H - (1/2)J$; $J < 0$) can be given as follows.[4]

$$R_2^{(1)} = \lambda + \eta \quad (1)$$

$$R_2^{(2)} = \lambda + \eta \quad (2)$$

$$\text{Where } \lambda = d[4J^{dd}(o) + 4\alpha^2 J^{cc}(o) + 3J^{dd}(\omega_H) + 3J^{cc}(\omega_H) + J^{dd}(\omega_H - \omega_N) + 3J^{dd}(\omega_N) + 6J^{dd}(\omega_H + \omega_N)] \quad (3)$$

$$\eta = 2\alpha d[4J^{cd}(o) + 3J^{cd}(\omega_H)] \quad (4)$$

$$d = (\mu_o/4\pi)^2 \gamma_H^2 \gamma_H^2 h^2/(80\pi^2 \gamma_{NH}^6) \quad (5)$$

$$\alpha = -(16\pi^2/3\mu_o)Bo(\sigma_{//} - \sigma_\perp)\gamma_{NH}^3/h\gamma_N \quad (6)$$

$J^{dd}(\omega)$, $J^{cc}(\omega)$ and $J^{cd}(\omega)$ are the spectral densities for dipolar autocorrelation, chemical shift anisotropy (CSA) autocorrelation, and dipolar-CSA cross correlation, respectively. B_0, γ_H, γ_N, h, μ_o, γ_{NH} and $(\sigma_{//} - \sigma_\perp)$ are the static magnetic field, gyromagnetic ratios of proton and nitrogen-15, Planck constant, magnetic permeability, the length of the NH bond and the chemical shift anisotropy (CSA) of the proton, respectively.

This treatment can be applied to the fully reduced cytochrome c_3. For an axially symmetric CSA tensor in a rigid body under isotropic rotational diffusion, these spectral densities are given by

$$J^{dd}(\omega) = J^{cc}(\omega) = J^{cd}(\omega)/P_2(\cos\theta) \quad (7)$$

$$P_2(\cos\theta) = (3\cos^2\theta - 1)/2 \quad (8)$$

where θ is the angle between the unique axes of the CSA and dipolar tensors.[6] Although Eq. (7) is no longer rigorous in the presence of the internal motion, it remains a good approximation provided θ is small and the relative orientation of the dipolar and CSA tensors is independent of the internal motion.[6,9] When it is the case, the superscripts in the spectral density function may be dropped, and $J(\omega)$ and η can be described as follows.[6]

$$J(\omega) = S^2 \tau_C / (1+\omega^2\tau_C^2) + (1-S^2)t / (1 + \omega^2\tau^2) \quad (9)$$

$$\eta = 2\alpha d\{4J(O) + 3J(\omega_H)\} P_2(\cos\theta) \quad (10)$$

where S, τ_c and τ are the generalized order parameter of the NH bond, the rotational correlation time of the protein and $(\tau_c^{-1}+\tau_e^{-1})^{-1}$, respectively. The time constant τ_e is determined by rapid internal motions. The angle θ should be small for the intrinsic proton CSA.[9] However, the ring current shift should be taken into account in the case of the imidazole coordinated to the heme iron.[12] Even if the size of the CSA in the ring current shift tensor is similar to the intrinsic proton CSA, θ is about 18º. Therefore, θ is still small. For a protein with the molecular weight of about 14000 such as cytochrome c_3, we can neglect $J(\omega_H)$ at the resonance frequency of 500 MHz as a first approximation.[9] The typical value of

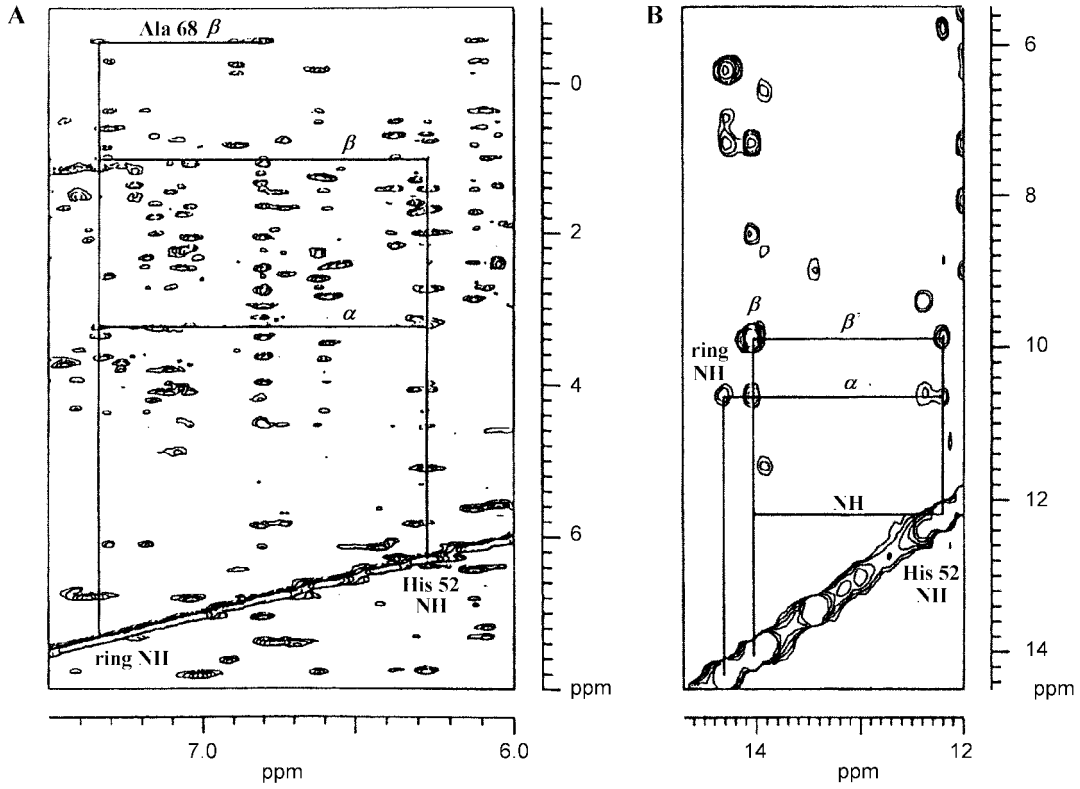

Figure 2. His 52 related sections of 2D NOESY spectra of uniformly ^{15}N labeled cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki F in the fully reduced (A) and fully oxidized (B) states. His 52 NH, ring NH, α, β and β' strand for the signals of the backbone NH, imidazole NH, αCH and two βCH$_2$ protones of His 52, respetively. Ala68β is the βCH$_3$ proton of Ala 68. The assignment of the signal on the line for a lateral line either on the top or at the bottom for a vertical line.

S^2 for the NH bond involved in the secondary structure in a protein is about 0.85.[6] Since the fluctuation of the imidazole group coordinated to the heme iron should be more suppressed than that of the peptide backbone and $\tau_e \leq \tau_c$, the contribution of the second term of $J(0)$ should be small. Thus,

$$J(0) \approx S^2\tau_C \tag{11}$$

Therefore, the transverse relaxation rates of the two components can be simplified as

$$R_2^{(1)} = 4d\{1 + \alpha^2 + 2\alpha P_2(\cos\theta)\}S^2\tau_c \tag{12}$$

$$R_2^{(2)} = 4d\{1 + \alpha^2 - 2\alpha P_2(\cos\theta)\}S^2\tau_c \tag{13}$$

There are also additional relaxation contributions such as dipole interactions with nearby protons, exchange broadening, field inhomogeniety and nonresolved J couplings from farther protons, which are the same for both peaks. They can be eliminated by subtraction. Subtraction of Eq. (13) from Eq. (12) gives

$$R_2^{(1)} - R_2^{(2)} = 16\tau_C d\{\alpha S^2 P_2(\cos\theta)\} \tag{14}$$

We need the rotational correlation time of cytochrome c_3 to calculate CSA. It was estimated from reported values for RNase H1,[13] calbindin,[14] calmodulin,[15] cytochrome c_{550}[16] and partially deleted cytochrome b_5[17] by the linear least-squares method, assuming that it is proportional to the molecular weight. The obtained value was 6.7 ± 0.8 ns at 30T. Since J_{NH}, is negative, the linewidths of the components I and 2 in the reduced state in Figure 1 are 25.6 and 29.6 Hz, respectively. From Eq. (14) and estimated t_C.

$$(\sigma_{//} - \sigma_{\perp})S^2P_2(\cos\theta) = -18 \pm 2 \text{ ppm} \tag{15}$$

Here, γ_{NH} was assumed to be 0.107 nm, which is the value for the hydrogen bonded imidazole NH determined by neutron diffraction.[18] The error comes from τ_C. $S^2P_2(\cos\theta)$ should be in the range of 1.0-0.7 in view of the assumption mentioned above. In the case of the imido proton of an uracil of a nucleoside analogue, $|\sigma_{//} - \sigma_{\perp}|$ was estimated to be 5.7 ppm on the basis of the spin-lattice relaxation time analysis.[2] The reduced |CSA| of the armide protons of savinase, ubiquitin and HU protein scattered in a wide range from 1 to 30 ppm.[7,8,9] The CSA observed for OH and NH groups in solid organic compounds is in the range of -10 ~ -35 ppm.[19,20,21] In the case of the fully oxidized cytochrome c_3, the electron spin-proton (Fe-^{1}H) interactions are additionally involved in the relaxation. The dipolar interaction will contribute to the interference term by altering the CSA. It should be noted that the CSA contribution of the electron spin is non-zero even if the g tensor is isotropic. There are three interference cross terms contributing to the transverse relaxation of the ^{15}N-coupled proton. The Hamiltonian can be written as.

$$H = H(^{15}\text{N} - {}^1\text{H dipole}) + H(^1\text{H CSA}) + H(\text{Fe-}^1\text{H dipole}) \tag{16}$$

In the Fe-^{1}H dipolar interaction, the electron spin relaxation is so short (about 2×10^{-2} S) that the effect on proton relaxation is negligible.[22] Here, the average electron spin polarization interacts with the proton. Therefore, the dipolar field due to the net paramagnetic moment induced by the external field changes the chemical shift of the proton depending on the orientation of the molecule. Consequently, the paramagnetic center adds a new contribution to the chemical shift tensor of the proton. Now, we can sum up the second and third terms of Eq. (16) as H (^{1}H CSA'), where CSA' is the chemical shift anisotropy term including a new contribution. Then, we can go back to Eqs. (1) and (2) with the CSA'. The effect of the electron spin-proton dipolar interaction on the chemical shift tensor can be estimated. A general formula for the dipolar shift induced by a paramagnetic center which has an axially symmetric g tensor, can be described as

$$\Delta B(\xi, W)/B_0 = (\mu_0/4\pi)\gamma^{-3}b^2(3kT)^{-1}S(S+1)$$
$$\{g_{//}^2\cos^2\xi(3\cos^2\chi-1) + g_\perp^2\sin^2\xi(3\sin^2\chi\cos^2\Omega-1)$$
$$+ 0.75(g_{//}^2 + g_\perp^2)\sin2\xi\sin2\chi\cos\Omega\} \quad (17)$$

where γ, β, S, $g_{//}$ and $g_\perp$ are the distance between the paramagnetic center and proton, Bohr magneton, total spin of the paramagnetic center, parallel and perpendicular components of the g tensor, respectively.[23] A positive value corresponds to a down field shift as in the definition of CSA. The angles are defined as $g = g_{//}\kappa\kappa + g_\perp(\lambda\lambda + \nu\nu)$, $B_0\cdot\kappa = B_0\cos\xi$ and $B_0\cdot\lambda = B_0\sin\xi$, $\bar{\gamma} = (\bar{\gamma}\cos\chi)\kappa + r\sin\chi$ $(\lambda\cos\Omega + \nu\sin\Omega)$ where κ, λ and ν stand for unit vectors of the axes of a cartesian coordinate, respectively. Specifically, κ is parallel to the direction of $g_{//}$ which is usually perpendicular to the heme plane. In the case of heme 2 of cytochrome c_3, the g tensor is not axially symmetric ($g_x^2 = 0.90$ $g_Y^2 = 3.88$ and $g_z^2 = 11.09$).[24] However, to estimate the qualitative effect of the heme iron on the chemical shift of the imido proton signal of the coordinated imidazole, $g_\perp^2 = (1/2)(g_x^2 + g_Y^2)$ was assumed. From the crystal structure, g_{FeH} and c for His-52 on heme 2 were found as 0.497 nm and 16°, respectively. The largest down-field shift is expected for $\xi = 13.31\times$ and $\Omega = 0°$ with 87.5 ppm, where the external magnetic field is almost parallel to γ_{FeH}. And the largest up-field shift is expected for $\xi = 77.3°$ and $\Omega = 180°$ with 13.0 ppm. In this case, the magnetic field is almost perpendicular to γ_{FeH}. Since the other principal value of the paramagnetic dipolar shift tensor is -10.0 ppm, this tensor is almost axially symmetric. The CSA in this tensor is about 100 ppm. The unique axis of this tensor is located in the plane made by the NH bond vector and the heme plane normal (the unique axis of the ring current shift tensor). The unique axis of the paramagnetic dipolar shift tensor is deviated from the NH vector and the heme plane normal by 20° and 16°, respectively, provided that the angle between the NH vector and the heme plane normal is 36°. Therefore, the unique axes of the proton CSA and the paramagnetic shift CSA are close to each other. However, the signs of these CSAs are opposite. The CSA of the imido proton of an imidazole without the contribution from the paramacnetic center would be in the range of 0 ~ -35 ppm (Eq. (15), and refs. 2, 7, 8, 9, 19, 20, 21). In view of its small size in comparison with that of the paramagnetic CSA, the total CSA will be dominated by the paramagnetic one on the appearance of a paramagnetic center, leading to the inversion of the sign of the total CSA from negative to positive.

The contribution of the interference term to the transverse relaxation in the fully oxidized state can be obtained using a simple model, namely Eqs. (1) and (2). The linewidths of the components 1 and 2 of the imido proton of His 52 in the fully oxidized state (Fig. 1) are 49.3 and 35.3 Hz, respectively. Since the oxidized hemes of cytochrome c_3 are paramagnetic, the paramagnetic relaxation should be taken into account. According to Goldman.

$$R_2'^{(1)} = 4d\{1 + \alpha'^2 + 2\alpha'P_2(\cos\theta')\}S^2\tau_C + R'' \quad (18)$$

$$R_2'^{(2)} = 4d\{1 + \alpha'^2 2\alpha'P_2(\cos\theta')\}S^2\tau_C + R'' \quad (19)$$

where R" is the paramagnetic relaxation due to the Fermi contact term[4]. α' and θ' are defined by CSA. The paramagnetic contribution from the iron is non-zero even if the g factor is isotropic. As discussed above, θ' should be smaller than 20°, which justifies the use of these equations. Subtraction of Eq. (19) from Eq. (18) gives

$$R_2'^{(1)}\ R_2'^{(2)} = l6-\tau_C d\{\alpha'S^2P_2(\cos\theta')\} \quad (20)$$

By using the estimated τ_C, the CSA' can be obtained as

$$(\sigma_{//} - \sigma_\perp)'S^2P_2(\cos\theta') = 63 \pm 8 \text{ ppm}$$

From the estimations described above, $S^2P_2(\cos\theta')$ should be in the range of 1.0-0.7. The obtained value is reasonable in view of the theoretical estimation. The calculation of CSA from the linewidths and the theoretical simulation lead us to the conclusion that the inversion of the linewidth of the doublet peaks of the ^{15}N-coupled imido proton of the coordinated imidazole group on the oxidation of the heme is induced by the electron spin proton dipolar interaction. The linewidths were obtained from the slices of 2D HMQC spectra because the NH signals were overlapped with many other proton signals in the spectrum of the reduced cytochrome c_3. The error could be larger than that obtained from one dimensional spectra. However, it would not change the conclusion mentioned above, because the error should be systematic. More general theory with an asymmetric chemical shift tensor was discussed by Goldman.[4] There are seven other doublets showing similar characteristics in the linewidths of the doublet peaks. They can be assigned to the imido protons of the other imidazoles coordinated to the four hemes. Since the orientation of the coordination of the imidazole is quite different for the four hemes, it can be safely concluded that the inversion of the linewidths of the ^{15}N coupled proton doublet is characteristic for a coordinated histidine.[25] Thus, this phenomenon can be used to assign the ^{15}N labeled imido proton signal of a coordinated imidazole group in a heme protein. The modification of the interference term also should be the

case with the ^{13}C-coupled proton signals of the chemical group coordinated to the heme in a heme protein. Therefore, this can be a general method to assign the NMR signals of the coordinated chemical groups in heme proteins. Furthermore, the electron spin-proton dipolar cross correlation could be another source of the structural parameters in the investigation of paramagnetic proteins.

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