Long-Range Structural Information in NMR Studies of Paramagnetic Molecules from Electron Spin−Nuclear Spin Cross-Correlated Relaxation

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Received April 19, 1999
Revised Manuscript Received June 8, 1999

Information on long-range order improves the structure determination of macromolecules by NMR, especially in the case of extended conformation or modular architecture.[1] Insertion of paramagnetic spin labels at specific sites allows the exploration of molecular structure at long distances.[2,3] The anisotropic magnetic susceptibility of the paramagnetic center induces shifts in the nuclear resonance frequencies[2] as well as a small degree of molecular alignment in the magnetic field. The alignment gives rise to measurable residual dipolar coupling,[3] dependent on the orientation of nuclear vectors with respect to a molecular coordinate system. Both paramagnetic shifts and residual dipolar couplings provide long-range orientational information which complements local structural constraints such as proton nOe and ^3J scalar coupling constants. In this paper, a novel source of long-range structural information in paramagnetic macromolecules is investigated. Structural constraints are obtained from the accurate measurement of cross-correlated relaxation rate constants involving the static magnetic moment of the paramagnetic center. An NMR experiment has been designed to quantitatively measure the effect on the ^{1}H spectrum of ^{15}N-labeled molecules.

Relaxation of nuclear spins in the presence of a paramagnetic center is influenced by time-modulated dipolar interactions with the magnetic moment of the electron spin *S*. When the electronic spin relaxation is fast compared to the molecular tumbling ($T_e \ll \tau_c$), the electron spin is decoupled from the nuclear spins and the line-broadening in the NMR spectrum is minimal. However, a dipolar interaction remains with the static electron magnetic moment $\vec{\mu}_S$, which depends on the relative populations of the electronic energy levels.[4] The induced *Curie spin relaxation*,[4a] or *susceptibility relaxation*,[4b] gives rise to differential line-broadening of the doublet lines in a scalar-coupled two-spin system, an effect recently observed in cytochrome c_3.[5] This paramagnetic cross-correlation between the ^{1}H-Curie spin and ^{1}H−^{15}N dipolar interactions is conceptually identical to ^{1}H CSA/^{1}H−^{15}N dipolar cross-correlated relaxation.[6]

In the high-temperature approximation $\vec{\mu}_S$ can be expressed in terms of the molecular susceptibility tensor $\bar{\chi}$ and the external magnetic field $\vec{B}$: $\vec{\mu}_S = \bar{\chi}\vec{B}$. For the simplified case of an isotropically tumbling rigid macromolecule with an isotropic susceptibility tensor χ the cross-correlated relaxation rate constant $\Gamma^{Curie,DD}_{H,H-N}$ is given by[7]

$$\Gamma^{Curie,DD}_{H,H-N} = K_{\text{cross}}\,\chi B_0 \tau_c \frac{P_2(\cos\theta)}{r^3_{H-S}} \qquad (1)$$

with the constant $K_{\text{cross}} = 2/5\ (\mu_o/4\pi)^2\ h/2\pi\ \gamma^2_H\gamma_N/\langle r_{H-N}{}^3\rangle$, θ is the angle defined by the triangle N−H−S, and r_{H-S} represents the distance between the amide proton and the electronic spin which is assumed to be localized on the iron (Figure 2). The magnetic susceptibility χ is given by: $\chi = \gamma^2_S(h/2\pi)^2 S(S+1)/3kT$, where *S* is the Curie-spin quantum number.[8] A large value of χ is expected for paramagnetic labels with a high spin quantum number *S*. For most systems, however, this simple relation is not strictly valid as the electronic energy levels are modified by the spin−orbit coupling.[2a,b]

The favorable r^{-3}_{H-S} dependence of the cross-correlated relaxation rate constant $\Gamma^{Curie,DD}_{H,H-N}$ compared to auto-correlated paramagnetic relaxation ($\propto r^{-6}_{H-S}$) or ^{1}H−^{1}H nOe ($\propto r^{-6}_{H-H}$) makes it an ideal candidate for obtaining long-range structural information in macromolecules. To investigate this cross-correlation effect experimentally, we have chosen the ferrocytochrome *c*′ from *Rhodobacter capsulatus* as a model system (Figure 1).[9] High-resolution crystal structures are available for the oxidized [10a] and a diamagnetic butylisocyanide-bound form of the molecule.[10b] In the reduced form the heme iron is in a high spin state ($S = 2$) as indicated by EPR and magnetic susceptibility measurements.[9] A diamagnetic form of cytochrome *c*′ ($S = 0$) is also obtained when complexed to carbon monoxide. Different experimental techniques have been proposed recently to measure ^{1}H CSA/^{1}H−^{15}N dipolar cross-correlation effects in proteins.[11] For the present study a sensitivity enhanced TROSY pulse sequence [12] was modified to include a transverse ^{1}H relaxation period prior to detection. The relaxation decay of the individual doublet lines described by the spin operators H^{α}_+ and H^{β}_+ is monitored by recording series of 2D ^{1}H−^{15}N correlation spectra. The pulse scheme and experimental details are provided as Supporting Information.

Intensity ratios were extracted for 91 well-resolved cross-peaks in the spectra of ferrocytochrome *c*′ and assigned to nuclear positions in the polypeptide sequence as reported previously.[9] Scalar J_{HN} coupling evolution during the relaxation period ensures monoexponential decay of the cross-peak intensities [6] as demonstrated in Figure 1. Relaxation rate constants $\Gamma_{H,H-N} = \Gamma^{Curie,DD}_{H,H-N} + \Gamma^{CSA,DD}_{H,H-N}$ are calculated from the measured intensity

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(1) (a) Tjandra, N.; Garrett, D. S.; Gronenborn, A. M.; Bax, A.; Clore, G. M. *Nat. Struct. Biol*. **1997**, *4*, 443−449. (b) Tjandra, N.; Omichinski, J. G.; Gronenborn, A. M.; Clore, G. M.; Bax, A. *Nat. Struct. Biol*. **1997**, *4*, 732−738. (c) Clore, G. M., Gronenborn, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5891−5898.

(2) (a) Berliner, L. J.; Reuben, J. *Biological Magnetic Resonance*; Plenum Press: New York, 1993; Vol. 12. (b) Bertini, I.; Luchinat, C. *NMR of paramagnetic molecules in biological systems*; The Benjamin/Cummings Publishing Company, Inc: Menlo Park, 1986. (c) Ramos, A.; Varani, G. *J. Am. Chem. Soc*. **1998**, *120*, 10992-10993. (d) Gochin, M.; Roder, H. *Protein Sci*. **1995**, *4*, 296−305.

(3) (a) Tolman, J. R.; Flanagan, J. M.; Kennedy, M. A. A.; Prestegard, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9279−9283. (b) Beger, R. D.; Marathias, V. S.; Volkman, B. F.; Bolton, P. *J. Magn. Reson*. **1998**, *135*, 256−259.

(4) (a) Gueron, M. *J. Magn. Reson*. **1975**, *19*, 58−66. (b) Vega, A. J.; Fiat, D. *Mol. Phys*. **1976**, *31*, 347−355.

(5) Ohmura, T.; Harada, E.; Fujiwara, T.; Kawai, G.; Watanabe, K.; Akutsu, H. *J. Magn. Reson*. **1998**, *131*, 367−372.

(6) Goldman, M. *J. Magn. Reson*. **1984**, *60*, 437−452.

(7) Ghose, R.; Prestegard, J. *J. Magn. Reson*. **1997**, *128*, 138−143.

(8) γ_H, γ_N, and γ_S are the gyromagnetic ratios of ^{1}H, ^{15}N, and the electron spin S, *h* is Planck's constant, μ_o is the magnetic field constant, and r_{H-N} is the proton−nitrogen distance, $P_2(x)$ is the second-order Legendre polynomial, τ_c is the correlation time of the rotational diffusion, *k* is the Boltzmann factor, and *T* is the sample temperature.

(9) (a) Caffrey, M.; Simorre, J.-P.; Brutscher, B.; Cusanovich, M. A.; Marion, D. *Biochemistry* **1995**, *34*, 5904−5912. (b) Tsan, P., Thèse de l'Université Joseph Fourier, 1998, Grenoble. (c) Tsan, P.; Caffrey, M.; Lawson-Daku, M.; Cusanovich, M.; Marion, D.; Gans, P. *J. Am. Chem. Soc*. **1999**, *121*, 1795−1805.

(10) (a) Tahirov, T. H.; Misaki, S.; Meyer, T. E.; Cusanovich, M. A.; Higuchi, Y.; Yasuoka, N. *J. Mol. Biol*. **1996**, *259*, 467−479. (b) Tahirov, T. H.; Misaki, S.; Meyer, T. E.; Cusanovich, M. A.; Higuchi, Y.; Yasuoka, N. *Nat. Struct. Biol*. **1996**, *3*, 459−464.

(11) (a) Tessari, M.; Vis, H.; Boelens, R.; Kaptein, R.; Vuister, G. W. *J. Am. Chem. Soc*. **1997**, *119*, 8985−8990. (b) Tjandra, N.; Bax, A. *J. Am. Chem. Soc*. **1997**, *119*, 8076−8082.

(12) Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12366−12371.

10.1021/ja991228t CCC: $18.00
Published on Web 08/05/1999

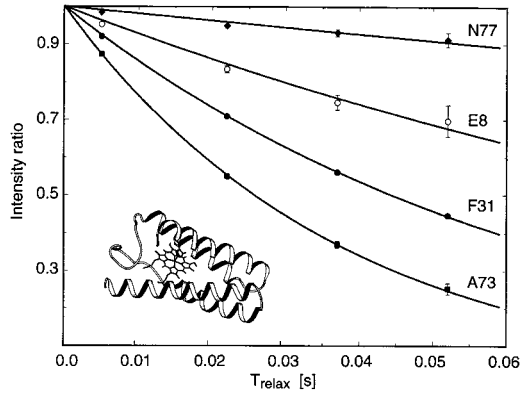

Figure 1. Intensity ratios $I(H^{\beta}_{+})/I(H^{\alpha}_{+})$ obtained for the amide protons of E8, F31, A73, and N77 in ferrocytochrome c' are plotted as a function of the relaxation time T_{relax}. For E8 $I(H^{\alpha}_{+})/I(H^{\beta}_{+})$ ratios are plotted as the cross-correlation effect is of opposite sign. The ratios were measured with the pulse scheme of Figure S1 on a Varian INOVA spectrometer (B_0 = 18.8 T). Relaxation rate constants were obtained from an exponential fit (solid lines) to the function $I(H^{\alpha}_{+})/I(H^{\beta}_{+}) = \exp\{\Gamma_{H,H-N}/2T_{relax}\}$. A ribbon representation of the four helix-bundle structural motif of cytochrome c' and the covalently attached heme is also shown.

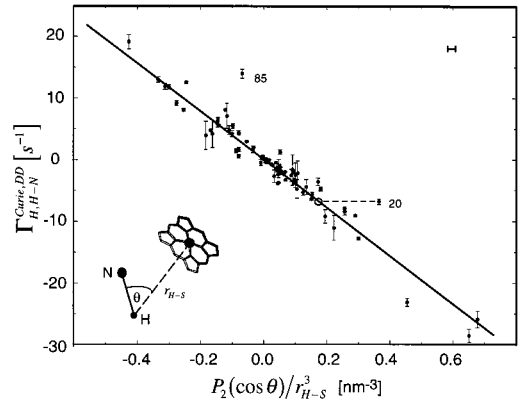

Figure 2. Correlation plot of measured $\Gamma^{Curie,DD}_{H,H-N}$ relaxation rate constants (B_0 = 18.8 T) and $P_2(\cos\theta)/r^3_{H-S}$ calculated from the crystal structure of ferricytochrome c'.[10a] The structural parameters θ and r_{H-S} are defined as shown in the lower left corner of the figure. The data cover a distance range of 9.5 Å $\leq r_{H-S} \leq$ 27.4 Å. For amide protons closer than 9.5 Å to the paramagnetic center no quantitative information was obtained due to paramagnetic line-broadening. The open circle corresponds to the $P_2(\cos\theta)/r^3_{H-S}$ value of S20 calculated from the butylisocyanide-bound form of cytochrome c'.[10b] The error bar indicated in the upper right corner corresponds to the standard deviation σ between the values obtained with the anisotropic and isotropic motional models.

ratios. As amide proton CSA tensors can significantly vary in a protein,[11] we have measured the diamagnetic contribution $\Gamma^{CSA,DD}_{H,H-N}$ on carbon monoxide-bound cytochrome c'. The expected linear B_0-field dependence was confirmed by repeating the experiment at three magnetic field strengths (Supporting Information).

The simple relation for $\Gamma^{Curie,DD}_{H,H-N}$ in eq 1 assumes the absence of significant local motion in the protein. Therefore, data from the N- and C-terminal residues have been excluded from further analysis as ^{15}N relaxation provided evidence for large amplitude nanosecond time-scale motion in these parts of the molecule.[9b] The $\Gamma^{Curie,DD}_{H,H-N}$ rate constants measured for the remaining 86 amide protons are plotted in Figure 2 as a function of $P_2(\cos\theta)/r^3_{H-S}$. The observed linear correlation is remarkable (r = 0.96) taking into account the possibility of small structural changes between the crystal and the solution structure or between the free and the carbon monoxide-bound forms of the protein. The largest deviations from linearity are observed for the amide protons of residues S20 and G85. In both cases a possible explanation for this discrepancy can be found when looking in detail at the structures.[10] The amide proton of G85 is involved in hydrogen bonding with the aromatic side chain of H89 which adopts two different conformations in the crystal structure, suggesting conformational disorder in this region of the molecule. The amide proton of S20 is close to the iron (r_{H-S} = 10.4 Å) and the angle θ differs by 8° between the two reported crystal structures. This small difference introduces a shift of $P_2(\cos\theta)/r^3_{H-S}$ from 0.37 to 0.17 nm^{-3}, which agrees very well with the measured relaxation rate constant (Figure 2). This example illustrates the sensitivity of the relaxation rate constants not only to the distance from the paramagnetic center but also to small angular variations.

In principle, anisotropic rotational diffusion and anisotropic susceptibility of the molecule can be explicitly taken into account for structural interpretation of the measured relaxation rate constants. Analytical expressions for the cross-correlated spectral density function $J^{aniso}_{H-S,H-N}(\omega)$ reported by Cuperlovic et al.[13] were used to investigate the effect of motional anisotropy on the geometrical factor $P_2(\cos\theta)/r^3_{H-S}$, as shown in the Supporting Information. The global error bar for $P_2(\cos\theta)/r^3_{H-S}$ in Figure 2 takes into account the inaccuracy introduced by the simplified motional model. Despite the significant anisotropic rotational diffusion of cytochrome c' (D_{xx} = 1.37, D_{yy} = 1.68, D_{zz} = 2.13 × 10^{7}s^{-1}),[9b] this uncertainty remains small compared to the $P_2(\cos\theta)/r^3_{H-S}$ distribution. For the study of biomolecules at high magnetic fields use of the simple isotropic diffusion model seems to be sufficient to derive structural information from the measurement of $\Gamma^{Curie,DD}_{H,H-N}$ relaxation rate constants. In the case of an anisotropic susceptibility tensor $\bar{\chi}$ the relaxation rate constants become additionally dependent on the $\bar{\chi}$ tensor orientation. However, as long as the magnetic anisotropy is at least an order of magnitude smaller than the average susceptibility, the correction to the relaxation rate constants as described by eq 1 is small and can be safely neglected.[4b]

Accurate measurement of the NMR parameters is the basis for their use as restraints in a structure refinement protocol. In the present case large $\Gamma^{Curie,DD}_{H,H-N}$ rate constants in the range from −12 to +6 s^{-1} with an average precision of ±0.5 s^{-1} are obtained for nuclear sites as far as 15 Å away from the paramagnetic center, thus providing important long-range structural information. As the NMR experiment is based on the sensitive TROSY pulse scheme we expect that accurate rate constants will be obtained even in the case of large biomolecular complexes. In general the magnetic susceptibility is not known and χ has to be treated as *one* additional floating parameter to be determined during structure refinement. The methodology presented in this paper is applicable to every molecule containing a paramagnetic spin label with a large magnetic susceptibility and fast electron spin relaxation. Curie spin−nuclear spin cross-correlated relaxation rate constants together with paramagnetic shifts and residual dipolar couplings provide a powerful source of long-range structural information in these systems.

Acknowledgment. We thank M. Caffrey and M. Cusanovich for the preparation of the ^{15}N-labeled cytochrome c' sample, and P. Tsan for providing resonance assignments prior to publication. This work was supported by the C.E.A., the C.N.R.S. (France), and M.S.I. (San Diego). This is publication no. 707 of the IBS- J.P. Ebel.

Supporting Information Available: Details of the NMR experiment, and plots of the magnetic field dependence of $\Gamma_{H,H-N}$ and the correlation of $J_{H-S,H-N}(0)/r^3_{H-S}$ calculated for isotropic and anisotropic rotational diffusion (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA991228T

(13) Cuperlovic, M.; Palke, W. E.; Geric, J. T.; Gray, G. A. *J. Magn. Reson.* Ser. B **1996**, *110*, 26−38.