Inorg. Chem. **2006**, *45*, 10016–10018

Inorganic Chemistry

Communication

Frequency-Switching Inversion-Recovery for Severely Hyperfine-Shifted NMR: Evidence of Asymmetric Electron Relaxation in High-Spin Co(II)

Erin A. Riley,[†] Amy K. Petros,[‡] Karen Ann Smith,[†] Brian R. Gibney,[‡] and David L. Tierney*,[†]

Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87131, and Department of Chemistry, Columbia University, New York, New York 10027

Received June 30, 2006

A new method for reliably measuring longitudinal relaxation rates for severely hyperfine-shifted NMR signals in aqueous solutions is presented. The method is illustrated for a well-defined cobalt tetracysteinate, with relevance to cobalt-substituted metalloproteins. The relaxation measurements are indicative of asymmetric electronic relaxation of the high-spin Co(II) ion.

Favorable electronic properties make high-spin Co(II) systems particularly amenable to study by nuclear magnetic resonance techniques, offering relatively narrow linewidths in conjunction with sizable chemical shift dispersion. Consequently, divalent cobalt has been used extensively as a spectroscopic surrogate for zinc[1] in sites as diverse as carbonic anhydrase,[2−7] aminopeptidases,[8,9] and the metallo-β-lactamases.[10−13] Recently, a significant amount of effort has been directed toward NMR characterization of cobalt-substituted derivatives of type-I blue copper proteins.[9,14−24]

Paramagnetic ^{1}H NMR in aqueous solution is often overwhelmed by the water resonance. Application of conventional (presaturation, WEFT and pulsed-field gradients)[25] or more sophisticated (IR-TOCSY[26] and SERF[27]) water-suppression techniques necessarily restricts the ability to measure reliable T_1 values for severely shifted species. Insufficient excitation bandwidth, limited to $(4^*\mathrm{PW})^{-1}$,[28] makes this particularly true for the β-carbon protons of a cobalt-bound cysteinate, which are typically shifted by 200−300 ppm. With this as motivation, we have developed a simple modification of the standard inversion recovery experiment, easily applied at high fields, which provides reliable relaxation data for resonances shifted far from the diamagnetic envelope in aqueous samples. We demonstrate the methodology here, using the pseudo-tetrahedral, tetracysteinate cobalt complex of the de novo peptide NH$_2$−KL**C**EGG**C**IG**C**GA**C**GGW−CONH$_2$ (**IGA**).[29]

The Cys β-proton region of Co-**IGA**'s NMR spectrum (Figure 1) consists of seven distinct resonances. The apparent "doublet" at 265 ppm (marked C, D in Figure 1) corresponds

---

* To whom correspondence should be addressed. E-mail: dtierney@unm.edu.

[†] University of New Mexico.

[‡] Columbia University.

(1) Maret, W.; Vallee, B. L. *Methods Enzymol.* **1993**, *226C*, 52−71.
(2) Luchinat, C.; Monnanni, R.; Sola, M. *Inorg. Chim. Acta* **1990**, *177*, 133−139.
(3) Moratal, J. M.; Donaire, A.; Salgado, J.; Martinez-Ferrer, M. J. *J. Inorg. Biochem.* **1990**, *40*, 245−253.
(4) Bertini, I.; Luchinat, C.; Pierattelli, R.; Vila, A. J. *Eur. J. Biochem.* **1992**, *208*, 607−615.
(5) Bertini, I.; Luchinat, C.; Pierattelli, R.; Vila, A. J. *Inorg. Chem.* **1992**, *31*, 3975−3979.
(6) Bencini, A.; Bertini, I.; Bini, T. *Inorg. Chem.* **1993**, *32*, 3312−3315.
(7) Bertini, I.; Jonsson, B.-H.; Luchinat, C.; R. Pierattelli, R.; Vila, A. J. *J. Magn. Reson. B* **1994**, *104*, 230−239.
(8) D'Souza, V. M.; Bennett, B.; Copik, A. J.; Holz, R. C. *Biochemistry* **2000**, *39*, 3817−3826.
(9) Harris, M. N.; Bertolucci, C. M.; Ming, L.-J. *Inorg. Chem.* **2002**, *41*, 5582−5588.
(10) Orellano, E. G.; Giardini, J. E.; Cricco, J. A.; Vila, A. J. *Biochemistry* **1998**, *37*, 10173−10180.
(11) Crowder, M. W.; Yang, K.-W.; Carenbauer, A. L.; Periyannan, G.; Seifert, M. E.; Rude, N. E.; Walsh, T. R. *J. Biol. Inorg. Chem.* **2001**, *6*, 91−99.
(12) Crawford, P. A.; Yang, K. W.; Sharma, N.; Bennett, B.; Crowder, M. W. *Biochemistry* **2005**, *44*, 5168−5176.
(13) Periyannan, G.; Costello, A. L.; Tierney, D. L.; Yang, K.-W.; Bennett, B.; Crowder, M. W. *Biochemistry* **2006**, *45*, 1313−1320.
(14) Piccioli, M.; Luchinat, C.; Mizoguchi, T. J.; Ramirez, B. E.; Gray, H. B.; Richards, J. H. *Inorg. Chem.* **1995**, *34*, 737−742.
(15) Salgado, J.; Jimenez, H. R.; Donaire, A.; Moratal, J. M. *Eur. J. Biochem.* **1995**, *231*, 358−369.
(16) Salgado, J.; Jimenez, H. R.; Moratal, J. M.; Kroes, S.; Warmerdam, G. C. M.; Canters, G. W. *Biochemistry* **1996**, *35*, 1810−1819.
(17) Vila, A. J.; Fernandez, C. O. *J. Am. Chem. Soc.* **1996**, *118*, 7291−7298.
(18) Fernandez, C. O.; Sannazzaro, A. I.; Vila, A. J. *Biochemistry* **1997**, *36*, 10566−10570.
(19) Donaire, A.; Salgado, J.; Moratal, J. M. *Biochemistry* **1998**, *37*, 8659−8673.
(20) Salgado, J.; Kalverda, A. P.; Diederix, R. E. M.; Canters, G. W.; Moratal, J. M.; Lawler, A. T.; Dennison, C. *J. Biol. Inorg. Chem.* **1999**, *4*, 457−467.
(21) Diederix, R. E. M.; Canters, G. W.; Dennison, C. *Biochemistry* **2000**, *39*, 9551−9560.
(22) Donaire, A.; Jimenez, B.; Moratal, J. M.; Hall, J. F.; Hasnain, S. S. *Biochemistry* **2001**, *40*, 837−846.
(23) Fernandez, C. O.; Niizeki, T.; Kohzuma, T.; Vila, A. J. *J. Biol. Inorg. Chem.* **2003**, *8*, 75−82.
(24) Dennison, C.; Sato, K. *Inorg. Chem.* **2004**, *43*, 1502−1510.
(25) Ming, L.-J. In *Physical Methods in Bioinorganic Chemistry*; L. Que, Jr., Ed.; University Science Books: Sausalito, CA, 2000.
(26) Huber, J. G.; Moulis, J.-M.; Gaillard, J. *Biochemistry* **1996**, *35*, 12705−12711.
(27) Hansen, D. F.; Led, J. J. *J. Mag. Res.* **2001**, *151*, 339−346.
(28) LaMar, G. N.; deRopp, J. S. In *Biological Magnetic Resonance*; Plenum Press: New York, 1993; Vol. 12, pp 1−78.
(29) Petros, A. K.; Shaner, S. E.; Costello, A. L.; Tierney, D. L.; Gibney, B. R. *Inorg. Chem.* **2004**, *43*, 4793−4795.

10.1021/ic061207h CCC: $33.50

© 2006 American Chemical Society
Published on Web 11/16/2006

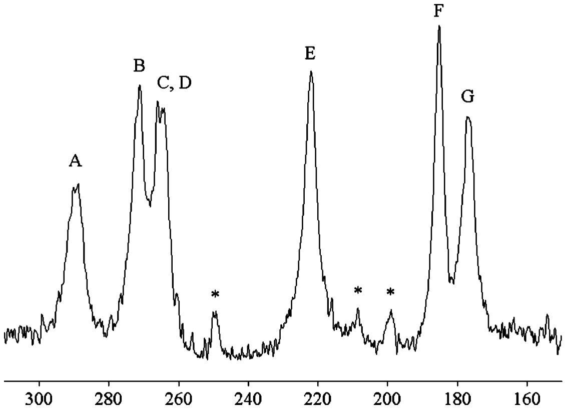

Figure 1. 500 MHz ^{1}H NMR spectrum of Co-**IGA** cysteine β-CH$_2$.

to two distinct resonances. Each peak integrates to one proton, except for the resonance at 222 ppm (E, two protons), accounting for all eight of the β-protons expected. The smaller resonances at 200, 210, and 250 ppm (marked with an asterisk), which integrate to less than 10% of the major resonances, may reflect the presence of a minor structural conformation of Co-**IGA**.[30]

Presaturation of the water signal provides effective solvent suppression, with the mildest hardware demands. The pulse sequence we present has the following form: RD-presat-VD-180°-τ-90°-AQ where RD is the recycle delay and AQ is data acquisition. What is unique here is that the presaturation pulse is applied on resonance with the water signal, and then, during the variable delay (VD), the transmitter is moved to the frequency where the inversion−recovery experiment (180-τ-90) is to be performed. By incorporating frequency switching, we are able to isolate the presaturation and inversion−recovery phases of the experiment. In order to attain steady-state suppression of the water signal, for all values of τ, the total time of the experiment was held fixed by keeping (VD + τ) equal to a constant. This results in a consistent baseline throughout the inversion−recovery experiment, greatly improving the precision of the T_1 measurement.

A stack plot demonstrating the application of this method to the resonances at 177 (G) and 185 (F) ppm is presented in Figure 2. These data demonstrate that as much as 96% inversion can be achieved with this method (see Figure S1). The insets to Figure 2 show the curve fits, clearly illustrating the lack of appreciable cross-relaxation, except at the longest τ values. Given the 25 μs 180° pulse length employed here, uniform excitation by the inversion pulse was limited to ±20 ppm from the transmitter frequency ($(4^*\mathrm{PW})^{-1} = 1$ kHz). Multiple experiments were necessary to acquire relaxation rates for all seven resonances. The results of these experiments are summarized in Table 1. The precision of this methodology is illustrated by the uncertainties, the largest of which is only 60 μs.

Relaxation measurements of this type, with at least one cysteinate coordinated to a Co(II) ion, are scarce in the literature. Of the handful available, two give only approximate values,[17,22] and three quote exact values[15,16,24] (one similar report on Ni(II) pseudoazurin[31] gives exact measurements for the β-^{1}H of a Ni(II)-bound Cys). It is impossible to draw comparisons to these reports, as the error involved in those measurements is not stated, but we must conclude that there is significant uncertainty in the single digit reported. All prior reports employed the nonselective superWEFT sequence (RD-180°-τ-90°-AQ),[32] using the initial slope method to extract T_1.[28] The use of superWEFT to measure relaxation rates for resonances so distant from the water signal is limited by excitation bandwidth. The initial slope method is used to eliminate contributions of cross-relaxation, and in order for it to give meaningful results, significant inversion must first be obtained. This requires that the resonance of interest be within the $(4^*\mathrm{PW})^{-1}$ limit, *based on the inversion pulse*. For a resonance with a chemical shift of 200 ppm, at 200 MHz (the *lowest* field used in previous reports), a 180° pulse length of *no more than* ~6 μs is required. A demonstration of this limitation, as defined by the 180° pulse length of our spectrometer, is presented in Figure S1, where only the peaks of interest are affected by both the inversion and observation pulses; the other resonances are only affected by the observation pulse. The method described here removes this limitation.

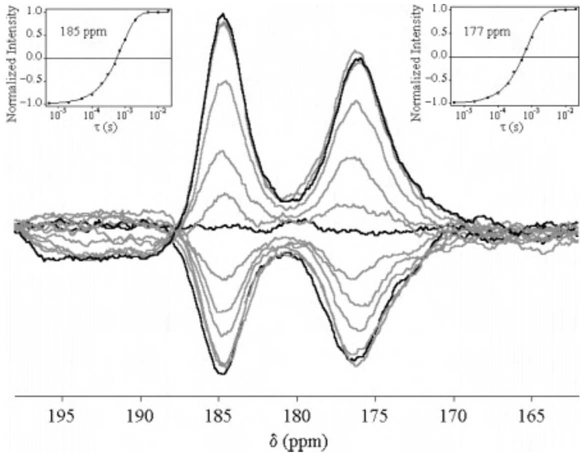

Figure 2. Inversion−recovery stack plot for resonances G (177 ppm) and F (185 ppm) in Figure 1. The corresponding curve fits (insets) are plotted on a log scale to emphasize the fit at short τ values.

Table 1. Longitudinal Relaxation Rates (T_1) for Co-**IGA** β-^{1}H Obtained with Frequency-Switching Inversion Recovery

peak (δ)	T_1 (ms)	R_1, θ_1 (T_1)[a]	R_2, θ_2[b]
G (177)	0.69 ± 0.05	3.85 Å, 90°	3.30 Å, −28°
F (185)	0.67 ± 0.06	3.83 Å, 87°	3.32 Å, −31°
E (222)	0.42 ± 0.01	3.55 Å, 60°	3.55 Å, −60°
D (264)	0.62 ± 0.06	3.78 Å, 84°	3.34 Å, −34°
C (266)	0.81 ± 0.06	3.96 Å, 105°	3.25 Å, −16°
B (271)	0.34 ± 0.02	3.42 Å, 45°	3.68 Å, −73°
A (289)	0.87 ± 0.04	4.00 Å, 105°	3.24 Å, −13°

[a] Co···H distances, assuming $\tau_c = 13$ ps. Co−S−C−H dihedral angles based on idealized Co−S−C angle of 112° (vide infra) and Co−S distance of 2.31 Å. [b] Predicted distance and dihedral angle of geminal partner, given the assumptions noted in footnote a.

In order to assign the resonances in Figure 1 to individual β-CH$_2$ pairs, saturation-transfer NOEs were measured. From

---

(30) Petros, A. K.; Reddi, A. R.; Kennedy, M. L.; Hyslop, A. G.; Gibney, B. R. *Inorg. Chem.* **2006**, in press.

(31) Dennison, C.; Sato, K. *Inorg. Chem.* **2002**, *41*, 6662−6672.

(32) Inubushi, T.; Becker, E. D. *J. Magn. Reson.* **1983**, *51*, 128−133.

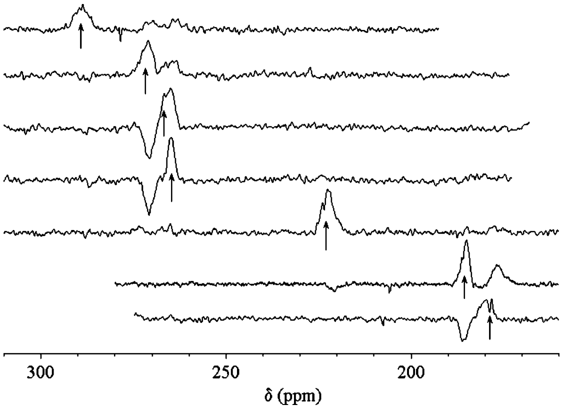

Figure 3. Saturation-transfer NOEs of cysteine β-^{1}Hs of Co-**IGA**. Irradiation frequency for each spectrum is marked with an arrow.

the NOE measurements (Figure 3), the β-^{1}H pairs are assigned as (A, D), (B, C), (E, E), and (F, G). The exact identity of the first two pairs is somewhat ambiguous, given the proximity of signals C and D; it is clear that A is paired with either C or D, and likewise, B. They are paired above with reference to their relative relaxation rates. On the basis of relations first described by Bertini et al. for Fe_4S_4 centers,[33,34] and later extended to cobalt-substituted blue copper proteins,[17−19,22,24,31] the NOE data imply that the Co−S−C−H dihedral angles are all close to 60°, as evidenced by the relatively small deviations of chemical shift ($\Delta\delta$) for a given β-CH_2 pair.

We now consider the dependence of the ^{1}H relaxation rates in Table 1 on the Co···H distances and dihedral angles. This system shows no evidence for thiolate lability on the NMR time scale, and its small size allows for moderately fast tumbling (MW $\approx$ 1578, $\tau_r \approx 2$ ns).[35] Consequently, the correlation time is expected to be defined by electron spin relaxation ($\tau_c = T_{1e}$). Prior analyses of contact shifts in Co-substituted blue copper proteins,[15,19,20,22,23] suggest that contact relaxation should be minimal.

Within the resolution of the data, the pair (E, E) must be exactly bisected by the Co−S−C plane ($\Delta\delta = 0$), and therefore, the Co···H distances must be the same. Our previous EXAFS data on Co-**IGA** suggests that the four cobalt−sulfur bonds are equivalent (2.31 Å), with little disorder.[29] In a molecule of this size, there is little driving force for large variations of the Co−S−C bond angles. We therefore adopt an idealized model of a cobalt tetracysteinate, setting the Co−S−C angle to 112°, consistent with the X-ray structure of Co-substituted azurin.[36] In the limit that (E, E) represents a symmetric geminal pair of β-CH_2 protons, they should each rest ~3.55 Å from the cobalt ion, with dihedral angles of ±60°.

Assuming that the Solomon−Bloembergen−Morgan (SBM)[37−39] relations hold for this system, the T_1 of 0.42 ms indicates a correlation time of 13 ps. SBM theory assumes a spherically distributed electronic relaxation rate (i.e., each proton feels the same correlation time), and therefore the value of 13 ps can then be used to extract the remaining Co···H distances (Table 1). The T_1 values in Table 1 range by a factor of 2.5 from shortest to longest, corresponding to a 17% spread in distance, based on an r^{-6} dependence of T_1. With the above model, the dihedral angle of a given proton can be extracted, given its distance.

The data in Table 1 present an interesting contradiction with this expectation. For example, the proton that gives rise to resonance G is predicted to be at a distance of 3.85 Å, corresponding to a dihedral angle of 90° (Table 1, column 3). On the basis of the model, its partner should then be at a distance of 3.30 Å, with a dihedral of −28° (Table 1, column 4). However, the NOE measurements show that proton G is partnered with proton F, which is predicted to be at 3.83 Å from the relaxation measurements, with an angle of 87°. Only one proton (B) is expected to be at a distance close to the 3.30 Å predicted for proton A's partner.

In fact, none of the predicted pairwise distances shown in Table 1 are consistent with the SBM model. While it is possible that the Co−S−C bond angles in Co-**IGA** deviate significantly from 112°, the probability of substantial differences is small. In order for the distances in Table 1 to match the pairs indicated by the NOE measurements, the Co−S−C bond angles would be required to be 122° (F, G), 112° (E, E), 127° (A, D), and 116° (B, C). The small size of Co-**IGA** is unlikely to be rigid enough to enforce such an unfavorable geometry.

An alternate explanation is perhaps the simplest: that SBM theory is insufficient to describe the relaxation behavior in this system. The SBM relations were originally derived for a spherically symmetric ground state in the absence of orbital angular momentum. In this system, with three unpaired electrons, the spherical symmetry implied by SBM is not observed. This failure of the point-dipole approximation implies that electron relaxation in this system is not spherically distributed, and an orientation-dependent correlation time is necessary to adequately interpret the paramagnetic relaxation enhancements. Such a situation could potentially arise for *any* system where the ground state is not of A symmetry or where significant spin−orbit coupling and/or Jahn−Teller effects are present.

Acknowledgment. This work was supported by the National Science Foundation (CHE-0518189 to D.L.T.) and the American Chemical Society−Petroleum Research Fund (41550-AC3 to B.R.G.).

Supporting Information Available: Experimental conditions and two figures showing the effect of excitation bandwidth on the NMR spectrum of Co-**IGA** (S1) and linear fits to the data in Figure 2 (S2) are included. This material is available free of charge via the Internet at http://pubs.acs.org.

IC061207H

---

(33) Bertini, I.; Capozzi, F.; Luchinat, C.; Piccioli, M.; Vila, A. J. *J. Am. Chem. Soc.* **1994**, *116*, 651−660.
(34) Karplus, M. *J. Am. Chem. Soc* **1963**, *85*, 2870−2871.
(35) Bertini, I.; Luchinat, C. *NMR of Paramagnetic Substances*; Elsevier: Amsterdam, 1996.
(36) Bonander, N.; Vanngard, T.; Tsai, L. C.; Langer, V.; Nar, H.; Sjolin, L. *Proteins* **1997**, *27*, 385−394.
(37) Solomon, I. *Phys. Rev.* **1955**, *99*, 559−565.
(38) Solomon, I.; Bloembergen, N. *J. Chem. Phys.* **1956**, *25*, 261−266.
(39) Bloembergen, N.; Morgan, L. O. *J. Chem. Phys.* **1961**, *34*, 842−850.