^{1}H and ^{15}N Hyperfine Shifts of Cytochrome *c*

Jonathan Boyd,[‡] Christopher M. Dobson,[‡] Artemiza S. Morar,[†] Robert J. P. Williams,[‡] and Gary J. Pielak*[,†]

Department of Chemistry, University of North Carolina Chapel Hill, North Carolina 27599-3290
Oxford Centre for Molecular Sciences
New Chemistry Laboratory, University of Oxford
South Parks Road, Oxford OX1 3QT, United Kingdom

Received June 16, 1999
Revised Manuscript Received August 4, 1999

Analysis of hyperfine NMR chemical shifts,[1] δ_{hf}, especially for protons, is an established method for probing the structure of redox proteins.[2] For cytochrome *c*, the redox-state shift, $\Delta\delta_{rdx}$, is the difference between the chemical shifts (in ppm) for identical nuclei in the paramagnetic low-spin Fe(III) species and the Fe(II) diamagnetic reference state, δ_{ox} and δ_{red}, respectively (eq 1).

$$\Delta\delta_{rdx} = \delta_{ox} - \delta_{red} = \delta_{hf} + \delta_{str} = (\delta_{con} + \delta_{lc} + \delta_{mc})_{hf} + \delta_{str} \quad (1)$$

$\Delta\delta_{rdx}$ has contributions from δ_{hf}, the shift from the unpaired electron in the Fe(III) form, and δ_{str}, the shift from redox-related changes in structure.[2d] δ_{hf} itself is composed of three terms. δ_{con} is the Fermi contact shift from direct delocalization of the unpaired electron spin on the Fe(III) ion to the heme, the directly linked thioether bonds of C14 and C17, and the axial heme ligands, H18 and M80.[3] δ_{lc}, which does not contribute in the absence of contact shifts, arises from the electron−nuclear dipolar interaction between the nucleus of an atom and the delocalized electron spin in its molecular orbitals. δ_{mc} arises from the electron−nuclear dipolar interaction between a nucleus and the unpaired electron on the Fe(III) ion, which has an anisotropic paramagnetic susceptibility. Analysis of >400 ^{1}H redox shifts for yeast cytochrome *c* shows that δ_{mc} dominates $\Delta\delta_{rdx}$ for protons.[2e] δ_{mc} also dominates ^{13}C redox shifts since there is excellent agreement between $\Delta\delta_{rdx}$ and δ_{mc} for a more limited number of side-chain carbons of horse cytochrome *c*.[4]

The amide proton and nitrogen are close in space, and, as shown in Figure 1A, the calculated δ_{mc} values for these nuclei are highly correlated. We have measured $\Delta\delta_{rdx}$ for backbone ^{1}H and ^{15}N nuclei of yeast iso-1-cytochrome *c*, specifically, the C102T variant.[5] $\Delta\delta_{rdx}$ and δ_{mc} for ^{1}H nuclei are in excellent agreement (Figure 1B), a finding consistent with our previous conclusions[2e] and confirming that structures of the two redox states of this protein are similar. The 13 ^{1}H redox shifts that deviate from the calculated δ_{mc} shifts by >|0.25| ppm either are heme ligands (C14, C17, M80) with known contributions from δ_{con},[3] are near these ligands (R13, L15, Q16), or have known contributions from δ_{str} (G29, H39, G41, A43, A51, D60, K79, A81).[2e,6a] Analysis of the ^{15}N data, however, reveals that many ^{15}N redox shifts show dramatic deviations from the calculated δ_{mc} values (Figure 1C). Unlike ^{1}H and ^{13}C nuclei, ^{15}N nuclei must experience additional contributions. Additional contributions to ^{15}N redox shifts are also evident for cytochrome b_5 and *Rhodobacter*

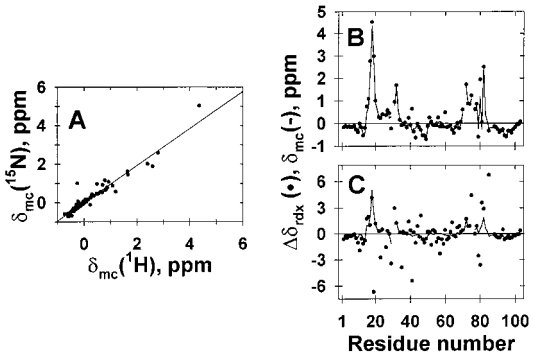

Figure 1. (A) Calculated $\delta_{mc}(^1H)$ versus calculated $\delta_{mc}(^{15}N)$ for 92 amide residues of yeast iso-1-cytochrome *c*, correlation coefficient squared, r^2, = 0.895. (B) Observed $\Delta\delta_{rdx}$ (●) and calculated $\delta_{mc}(^1H)$ (−) for 92 amide protons measured at 750 MHz, $r^2 = 0.949$. (C) $\Delta\delta_{rdx}$ (●) and calculated $\delta_{mc}(^{15}N)$ (−) for 92 amide nitrogens measured at 76 MHz, $r^2 = 0.087$. The 1.23-Å X-ray structure of iso-1-cytochrome *c* (1ycc.pdb)[6a] has coordinates for 107 backbone amides. Residues −4 to 1, which are known to be flexible, were not included in the calculations, six sets of ^{1}H and ^{15}N resonances (E21, H33, G83, G84, K86, and K87) remain unassigned and the sequence contains four proline residues. Amide protons were added to the X-ray structure, in the peptide plane, with XPLOR,[12] followed by energy minimization with the backbone heavy atoms constrained to their initial positions. The axial and rhombic anisotropies of the effective **g**-tensor were from ref 2e ($\mathbf{g}_{ax} = 4.58$ and $\mathbf{g}_{rh} = -2.31$). A diagonal, heme-based, right-handed, orthogonal axis system was constructed for the paramagnetic susceptibility tensor employing the heme pyrrole nitrogen atoms.[2b] This system has the Fe atom at the origin, the *x*-axis through pyrrole nitrogens 21 and 23, the *y*-axis nearly through nitrogens 22 and 24, and the *z*-axis perpendicular to the heme. The orientation of the calculated paramagnetic susceptibility tensor was subjected to a rotational transformation $R(\varphi,\theta,\phi)$, where φ, θ, and ϕ are Euler angles that optimize the agreement between the calculated and observed ^{1}H redox shifts. The angles (339°, 93.5°, and 121°, respectively) were determined by minimizing the function $F(\varphi,\theta,\phi) = \sum_{i=1}^{92}[(\delta_{mc})_i - (\Delta\delta_{rdx})_i]^2$. The eigenvector matrix defining this orientation is

x	*y*	z
0.3402	0.7857	0.5166
−0.1399	−0.5009	0.8541
0.9299	−0.3628	−0.0605

An additional restraint was imposed upon the orientation with the requirement that it agree with the small ^{15}N−^{1}H residual dipolar couplings arising from magnetic orientation of the oxidized species.[13] The *F*-test[14] indicates that this refinement significantly improves the ^{1}H data (*F*-value 116.9) but not the ^{15}N data (*F*-value 1.35). The δ_{mc} calculations[1] neglect the contribution from partial magnetic orientation.[15] This is justified since we observed no field dependence [at 500 MHz, r^2 for the calculated and observed ^{1}H and ^{15}N data is 0.952 and 0.087, respectively, for 81 resonances], consistent with an estimate (8×10^{-3}) for the orientation dependent term in eq 1 of ref 15, calculated employing the measured isotropic susceptibility (50.7×10^{-26} J T^{-2})[16] and an estimated total susceptibility anisotropy.[13]

* Corresponding author. E-mail: gary_pielak@unc.edu.
† University of North Carolina.
‡ University of Oxford.

(1) McConnell, H. M.; Chesnut, D. B. *J. Chem. Phys.* **1958**, *28*, 107. McConnell, H. M.; Robertson, R. E. *J. Chem. Phys.* **1958**, *29*, 1361. Kurland, R. J.; McGarvey, B. R. *J. Magn. Reson.* **1970**, *2*, 286−301. Buckingham, A. D.; Stiles, P. J. *Mol. Phys.* **1972**, *24*, 99.

(2) (a) *NMR of Paramagnetic Molecules: Principles and Applications*; La Mar, G. N., Horrocks, W. D., Jr., Holm, R. H., Eds.; Academic Press: New York, 1973. (b) Williams, G.; Clayden, N. J.; Moore, G. R.; Williams, R. J. P. *J. Mol. Biol.* **1985**, *183*, 447−460. (c) Timkovich, R.; Cai, M. *Biochemistry* **1993**, *32*, 11516−11523. (d) Pielak, G. J.; Auld, D. S.; Betz, S. F.; Hilgen-Willis, S. E.; Garcia, L. L. In *Cytochromes c: A Multidisciplinary Approach*; Scott, R. A., Mauk, A. G., Eds.; University Science Books: Sausalito, CA, 1996; pp 203−284. (e) Gao, Y.; Boyd, J.; Pielak, G. J.; Williams, R. J. P. *Biochemistry* **1991**, *30*, 1928−1934.

(3) Turner, D. L. *Eur. J. Biochem.* **1993**, *211*, 563−568.

(4) Turner, D. L.; Williams, R. J. P. *Eur. J. Biochem.* **1993**, *211*, 555−562.

(5) Morar, A. S.; Kakouras, D.; Young, G. B.; Boyd, J.; Pielak, G. J. *J. Biol. Inorg. Chem.* **1999**, *4*, 220−222.

(6) (a) Brayer, G. D.; Murphy, M. E. P. In *Cytochrome c: A Multidisciplinary Approach*; Scott, R. A., Mauk, A. G., Eds.; University Science Books: Sausalito, CA, 1996; pp 103−166. (b) Fetrow, J. S.; Baxter, S. M. *Biochemistry* **1999**, *38*, 4480−4492.

10.1021/ja9920361 CCC: $18.00
Published on Web 09/22/1999

capsulatus cytochrome *c*,[7] suggesting that this is a general characteristic of heme proteins and maybe many others.

The additional contributions could arise from δ_{con}, δ_{lc}, and δ_{str}. Contributions from δ_{con} and δ_{lc} are likely to be most significant for ^{15}N nuclei close to the heme. Such delocalization of heme electron density is predicted from theories of electron transfer,[8] but it is unlikely that these terms are large enough to explain the majority of the shifts observed in this work for several reasons. First, the largest additional contributions to the ^{15}N redox shifts are not from residues whose amide protons experience a contact shift. Second, many of the ^{15}N nuclei with the largest additional contributions are far from the heme. ^{15}N nuclei are, however, exquisitely sensitive to their environment; changes of <0.2 Å in hydrogen bond length can alter amide ^{15}N chemical shifts by several ppm,[9] the magnitude of the effect observed here. Although the X-ray structures of the different redox states of cytochrome *c* are closely similar,[6a] such movements would be within the limits of the resolution of the structural analysis. We conclude that the additional contributions to the ^{15}N data come from δ_{str}, and they most likely arise through small changes in hydrogen bonding,[9] although there may also be contributions from conformational changes in some cases. In support of these ideas, ^{15}N nuclei in the N-terminal helix (residues 2−16) and the C-terminal helix (residues 87−103), the most highly conserved and most rigid regions of the protein,[6] do not show large additional contributions.

^{15}N nuclei with the largest δ_{str} contributions mostly cluster on the surface near the exposed heme edge, which serves as the protein binding site,[10a,b] and near the heme-propionate-binding amino acids at the back of the molecule (Figure 2). A similar pattern is seen for cytochrome b_5 (Figure 2), where ^{15}N nuclei with the largest δ_{str} contributions cluster near the heme edge at the cytochrome *c* binding site.[10c] These data show that subtle changes to the binding surface result from the change in the oxidation state. Moreover, the findings suggest that electron transfer within proteins could be coupled to subtle, yet extensive, changes in hydrogen bonding, providing a mechanism for transmitting electronic changes at the heme group to cooperative structural changes at the protein surface. Both changes could occur by tunneling[11] since the N···O distance changes by <0.2 Å. In summary, analysis of ^{15}N redox shifts is a highly sensitive method for monitoring small cooperative structural and energetic alterations that could be of great importance not only for interprotein electron transfer but also for other changes in proteins.

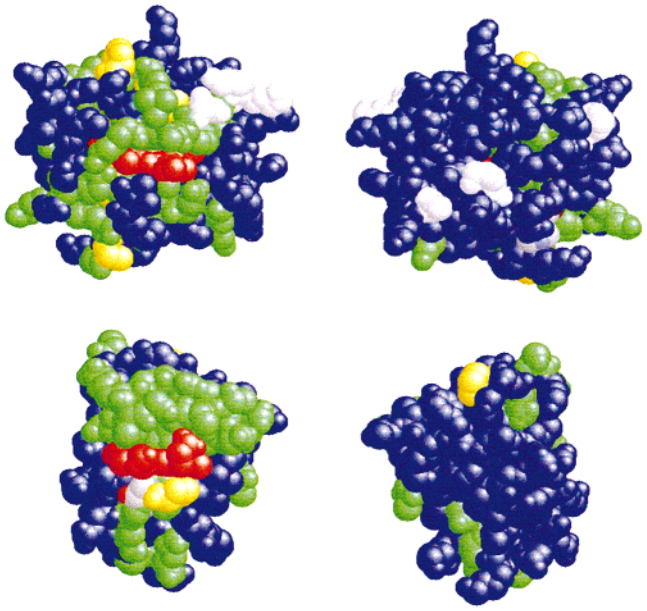

Figure 2. Space-filling models of yeast iso-1-cytochrome *c* (top) and bovine cytochrome b_5 (below) [blue, residues where $\Delta\delta_{rdx}(^{15}N) - \delta_{mc}(^{15}N) < |0.75|$ ppm; green, $>|0.75|$ ppm; red, heme; yellow, proline; white, unassigned]. The right-hand structures are rotated 180° about the vertical axis to show the faces remote from the binding site. δ_{mc} for cytochrome b_5 was calculated as described in the caption to Figure 1 by using coordinates from the 1.5-Å X-ray structure (3b5c.pdb) with added protons and the published assignments[8a] and **g**-tensor anisotropies ($\mathbf{g}_{ax} = 5.25$ and $\mathbf{g}_{rh} = -2.31$).[17] φ, θ, and ϕ are 331°, 72°, and 182°, respectively. The *F*-test[14] for 89 resonances indicates that this refinement significantly improves the ^{1}H data (*F*-value 93.6) but not the ^{15}N data (*F*-value 1.65). r^2 values for plots of $\Delta\delta_{rdx}$ versus δ_{mc} are 0.981 and 0.408 for ^{1}H and ^{15}N, respectively.

Acknowledgment. This work is supported by NIH Grant GM42501. G.J.P. thanks the Underwood Fund of the BBSRC for making this collaboration possible. A.S.M. was partially supported by a Ledoux/Dobbins Fellowship. The OCMS is supported by the BBSRC, EPSRC, and MRC.

Supporting Information Available: ^{1}H and ^{15}N redox shifts (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA9920361

(7) (a) Guiles, R. D.; Basus, V. J.; Sarma, S.; Malpure, S.; Fox, K. M.; Kuntz, I. D.; Waskell, L. *Biochemistry* **1993**, *32*, 8329−8340. (b) Tsan, P.; Caffrey, M.; Daku, M. L.; Cusanovich, M.; Marion, D.; Gans, P. *J. Am. Chem. Soc.* **1999**, *121*, 1795−1805.

(8) Beratan, D. N.; Betts, J. N.; Onuchic, J. N. *Science* **1991**, *252*, 1285−1288.

(9) de Dios, A. C.; Pearson, J. G.; Oldfield, E. *Science* **1993**, *260*, 1491−1496. Facelli, J. C.; Pugmire, R. J.; Grant, D. M. *J. Am. Chem. Soc.* **1996**, *118*, 5488−5489. Walling, A. E.; Pargas, R. E.; de Dios, A. C. *J. Phys. Chem.* **1997**, *101*, 7299−7303.

(10) (a) Pelletier, H.; Kraut, J. *Science* **1992**, *258*, 1748−1755. (b) Miller, M. A.; Geren, L.; Han, G. W.; Saunders: A.; Beasley, J.; Pielak, G. J.; Durham, B.; Millett, F.; Kraut, J. *Biochemistry* **1996**, *35*, 668−673. (c) Mauk, A. G.; Mauk, M. R.; Moore, G. R.; Northrup, S. H. *J. Bioeng. Biomem.* **1995**, *27*, 311−330.

(11) Kohen, A.; Klinman, J. P. *Acc. Chem. Res.* **1998**, *31*, 397−404. Williams, R. J. P. *J. Solid State Chem.* **1999**, *145*, 488−495.

(12) Brunger, A. T. *X-PLOR Version 3.1: A system for X-ray Crystallography and NMR*; Yale University Press: New Haven, CT, 1992.

(13) Boyd, J.; Pielak, G. J.; Morar, A. S.; Dobson, C. M., manuscript in preparation.

(14) Devore, J. L. *Probability and Statistics for Engineering and the Sciences*, 3rd ed.; Duxbury Press: Belmont, CA, 1991; pp 487−493.

(15) Bertini, I.; Felli, I. C.; Luchinat, C. *J. Magn. Reson.* **1998**, *134*, 360−364.

(16) Ångstrom, J.; Moore, G. R.; Williams, R. J. P. *Biochim. Biophys. Acta* **1982**, *703*, 87−94.

(17) Veitch, N. C.; Whitford, D.; Williams, R. J. P. *FEBS Lett.* **1990**, *269*, 297−304.