bases for the phenomenon we have observed. Since the coexistence of multiple, slowly interconverting conformations for a DNA sequence under biological conditions is unprecedented,[19] its generality and relevance are of considerable interest.

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(19) Slow equilibration between two conformations of the oligonucleotide d(TCGA) has previously been observed by NMR spectroscopy under conditions of low temperature (<10 °C) and very low ionic strength (no added salt).[20]

(20) Reid, D. G.; Salisbury, S. A.; Brown, T.; Williams, D. H. *Biochemistry* **1985**, *24*, 4325–4332.

^1H NMR Probe for Hydrogen Bonding of Distal Residues to Bound Ligands in Heme Proteins: Isotope Effect on Heme Electronic Structure of Myoglobin

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The ligand-binding properties of heme proteins depend critically on the specific interaction of the axial iron ligands (proximal histidine, bound ligand) with the polypeptide matrix.[2] Steric interplay with distal residues can influence the orientation of bound O_2 or induce tilting of CO or CN^- which would otherwise bind perpendicularly to the heme.[3-6] Hydrogen-bonding interactions can involve either the proximal histidyl imidazole[2,3,7] or a direct interaction between a protic distal side chain ligand and a bound ligand.[2,8,9] The ability to detect the influence of such H-bonding interactions in heme proteins relies primarily on the observation of solvent isotope effects on vibrational frequency[10,11] or ESR line width.[12] While these techniques clearly detect localized effects due to H-bonding, they are generally unable to identify the participating proton. H-bonding by an axial histidyl imidazole or to a bound ligand are detectable by X-ray and neutron diffraction,[3,8] but in the presence of several such interactions, these techniques cannot interpret their functional consequences. ^1H NMR is well suited for both the rapid and direct detection of H-bonding and the identification of the participating residue. While the task poses formidable experimental problems for a diamagnetic heme protein, the increased resolution for heme cavity residues in paramagnetic complexes and the exquisite sensitivity of the hyperfine shift to small structural perturbations set ideal conditions for elucidating the H-bonding interactions.[13] We demonstrate herein an isotope effect on the *electronic structure of the heme* in the low-spin, ferric cyanide complex of sperm whale myoglobin, metMbCN, which can be uniquely attributed to an H-bond between the distal (E7) histidyl imidazole and the bound ligand.

The low field portion of the 500-MHz ^1H NMR spectrum of metMbCN in 90% ^1H_2O, 10% ^2H_2O is illustrated in Figure 1A; the previously assigned heme peaks relevant to the work are indicated,[14] with labile proton peaks *A*, *B*, and *C* arising from the distal His E7 ring N_3H, proximal His F8 ring N_1H, and peptide

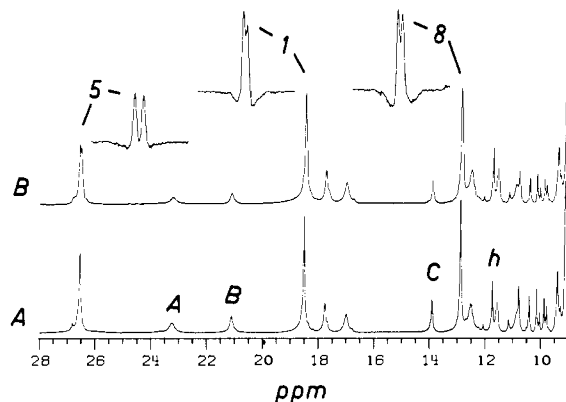

Figure 1. Downfield hyperfine shifted region of the 500-MHz ^1H NMR spectrum of sperm whale metMbCN at pH 9.2, 30 °C. (A) In 90:10 $H_2O/^2H_2O$. Peaks are labeled as reported previously.[15-17] *A*, distal His (64) ring N_3H; *B*, proximal His (93) ring N_1H; *C*, proximal His NH; *h*, distal His C_4H. The water resonance was suppressed by using an off-acquisition saturation pulse. (B) In 49:51 $H_2O/^2H_2O$ (pH 9.2). The expanded plots show the three resolved heme methyl resonances after resolution enhancement (sine-bell); the vertical scale is arbitrary, frequency scale magnified 5 times. Asymmetry in the expanded peaks is due to overlapping lines of different width. Note that A, B, C, and h remain single lines.

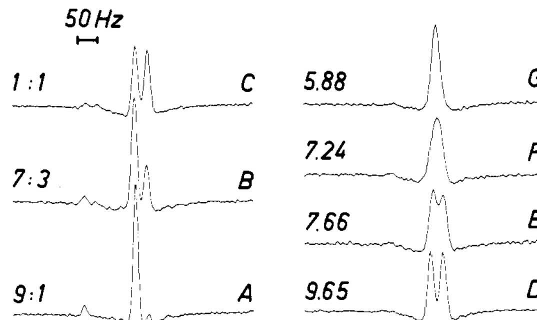

Figure 2. Heme 5-methyl signal(s) of metMbCN at 30 °C. (A–C) At pH 9.2 as a function of solvent composition ($^1H_2O/^2H_2O$): (A) 9:1, (B) 7:3, (C) 1:1. (D–G) In 1:1 $^1H_2O/^2H_2O$ as a function of pH: (D) pH 9.65, (E) pH 7.66, (F) pH 7.24, (G) ph 5.88. The vertical scaling was accomplished by reference to unaltered γ-CH_3 of Ile-99 at −3.2 ppm (not shown). The spectrum at pH 7.24 (F) corresponds to the coalescence of the two component peaks. For clarity, the 5-methyl signals are centered with respect to trace D in D–G in order to compensate for the effect of the titration of His 97 on the chemical shifts.

(1) Present address: Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.

(2) (a) Moffat, K.; Deatherage, J. F.; Seybert, D. S. *Science (Washington, D.C.)* **1979**, *206*, 1035–1042. (b) Traylor, T. G.; Traylor, P. S. *Annu. Rev. Biophys. Bioeng.* **1982**, *11*, 105–127, and references therein.

(3) Phillips, S. E. V. *J. Mol. Biol.* **1980**, *142*, 531–554.

(4) (a) Heidner, E. J.; Ladner, R. C.; Perutz, M. F. *J. Mol. Biol.* **1976**, *104*, 707–722. (b) Baldwin, J. M. *J. Mol. Biol.* **1980**, *136*, 103–128. (c) Hanson, J. C.; Schoenborn, B. P. *J. Mol. Biol.* **1981**, *153*, 117–124. (d) Powers, L.; Sessler, J. L.; Woolery, G. L.; Chance, B. *Biochemistry* **1984**, *23*, 5519–5523.

(5) (a) Bretscher, P. A. *Nature (London)* **1968**, *219*, 606–607. (b) Hendrickson, W. A.; Love, W. E. *Nature (London)* **1971**, *232*, 197–203. (c) Deatherage, J. F.; Loe, R. S.; Anderson, C. M.; Moffat, K. *J. Mol. Biol.* **1976**, *104*, 687–706.

(6) Scheidt, W. R.; Gouterman, M. In *Iron Porphyrins, Part I*; Lever, A. B. P., Gray, H. B., Eds.; Addison-Wesley: Reading, MA, 1983; pp 89–139.

(7) Stein, P.; Mitchell, M.; Spiro, T. G. *J. Am. Chem. Soc.* **1980**, *102*, 7795–7797.

(8) Phillips, S. E. V.; Schoenborn, B. P. *Nature (London)* **1981**, *292*, 81–82.

(9) Mispelter, J.; Momenteau, M.; Lavalette, D.; Lhoste, J. M. *J. Am. Chem. Soc.* **1983**, *105*, 5165–5166.

(10) Yoshikawa, S.; O'Keeffe, D. H.; Caughey, W. S. *J. Biol. Chem.* **1985**, *260*, 3518–3528.

(11) (a) Barlow, C. H.; Ohlsson, P.-I.; Paul, K.-G. *Biochemistry* **1976**, *15*, 2225–2229. (b) Smith, M. J.; Ohlsson, P.-I.; Paul, K.-G. *FEBS Lett.* **1983**, *163*, 303–305. (c) Satterlee, J. D.; Erman, J. E. *J. Am. Chem. Soc.* **1984**, *106*, 1139–1140.

(12) Walker, F. A.; Bowen, J. *J. Am. Chem. Soc.* **1985**, *107*, 7632–7635.

(13) La Mar, G. N. In *Biological Applications of Magnetic Resonance*; Shulman, R. G., Ed.; Academic: New York, pp 305–343.

(14) (a) Mayer, A.; Ogawa, S.; Shulman, R. G.; Yamane, T.; Cavaleiro, J. A. S.; Rocha Gonsalves, A. M. d'A.; Kenner, G. W.; Smith, K. M. *J. Mol. Biol.* **1974**, *86*, 749–756. (b) Lecomte, J. T. J.; La Mar, G. N. *Eur. Biophys. J.* **1986**, *13*, 373–381.

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NH, respectively.[15,16] Except for the presence of exchangeable proton resonances, the spectrum is very similar but not quite identical with that reported in ^2H_2O.[14] In a 50:50 mixture of ^1H_2O and ^2H_2O (Figure 1B), the spectrum corresponds to a 1:1 superposition of the subspectra recorded in the isotopically pure solvents. It is obvious that most but not all nonexchangeable proton signals coincide: the three heme methyl peaks yield double lines as shown in the expansion of Figure 1B. The relative splittings of the 5-, 1-, and 8-methyl peaks are 32, 16, and 18 Hz, respectively. Thus, the isotope composition of the solvent exerts an influence on the electronic structure of the heme, and the two species differentiated by the solvent isotopes are in slow exchange on the NMR time scale ($\ll 2 \times 10^2\ s^{-1}$) at 30 °C and pH 9.2.

The response of the two 5-methyl peak intensities to variable solvent isotope composition is illustrated by the three ^1H_2O:^2H_2O ratios shown in Figure 2: 90:10 (A), 70:30 (B), and 50:50 (C). In any solvent mixture, there are two and only two lines per heme methyl group, and their relative intensity is identical with the bulk ^1H:^2H solvent composition. This immediately allows the conclusion that *the isotope perturbation on the heme electronic structure is due exclusively to a single* $^1H/^2H$ *exchange site in the protein.* The low field component for each heme methyl corresponds to the "^1H" form, while the high field component arises from the "^2H" form in each case. Splittings for other resonances are not resolved.

Logical candidates for this unprecedented isotope effect on the heme electronic structure are heme cavity labile protons: proximal His ring or peptide protons (*B* or *C*) and distal His ring proton (*A*). Specific assignment can be effected on the basis of the known exchange rates for *A*, *B*, and C.[16] When the bound ^1H or ^2H exchanges with bulk solvent at a rate much faster than the chemical shift separation, the two subspectra must coalesce into one averaged spectrum. The effect of pH at 30 °C is displayed in traces D–G of Figure 2, which demonstrate that the dynamic collapse of the two 5-methyl signals is acid-catalyzed. Furthermore, the lines remain well-resolved and sharp up to pH 10.4. Only the distal His ring NH exhibits a compatible exchange behavior.[16] With use of the equation for a two-site exchange process,[17] analysis of the line width of the collapsed line at pH 7.24 yields an exchange rate of $\sim 10^2\ s^{-1}$, which is of the order of that determined for the distal His ring NH by saturation transfer from bulk solvent.[16,18] Differential paramagnetic relaxation has already demonstrated that the His E7 ring (peak *A*) must be within 4.2 Å of the iron and thus close to the bound cyanide.[16]

We conclude that the perturbation of the heme electronic structure arises directly from the isotope effect of the H bond between the distal His E7 and the bound ligand.[19] Since such an H bond is present in MbO_2[8] and absent in MbCO,[20] our NMR data indicate that, regarding distal interactions, cyanide is a better model for O_2 than for CO, even though the conformational tendencies of CN^- more closely resemble that of CO. The relative magnitudes of the isotope splitting and the fact that only heme resonances exhibit such splitting demand that the contact, and not the dipolar, contribution[13] to the hyperfine shift be sensitive to the $^1H/^2H$ replacement. Since the resolved "^1H" heme methyl shifts are all downfield of the "^2H" components, it appears that the heme in-plane asymmetry is larger in ^1H_2O than in ^2H_2O. The observed shifts confirm the direct influence of the distal ligand on the heme electronic structure and provides important evidence for its contribution to the asymmetry of the unpaired spin distribution.[13,21] This can be rationalized by the observation that the CN^- is tilted from the heme normal[5] and suggests that the differential $^1H/^2H$ bonding either induces an electronic perturbation in the Fe–CN bond or causes a small change in tilt angle. The absence of an isotope effect on the bound cyanide stretching frequencies in metMbCN[5] favors the latter interpretation. The effect of H bonding on the heme methyl contact shift pattern requires reassessment of the simple picture where only the proximal imidazole orientation modulates the in-plane asymmetry.[21] A more quantitative interpretation awaits the availability of a suitable X-ray structure of metMbCN.

The observation of similar, but highly differential, solvent isotope splittings of heme resonances in a variety of ferric cyanide ligated derivatives of other myoglobins, hemoglobins, and peroxidases indicates that the present NMR method provides a new probe of H bonding in heme proteins which will have broad applicability.

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(15) Sheard, B.; Yamane, T.; Shulman, R. G. *J. Mol. Biol.* **1970**, *53*, 35–48.
(16) Cutnell, J. D.; La Mar, G. N.; Kong, S. B. *J. Am. Chem. Soc.* **1981**, *103*, 3567–3572.
(17) Sandström, J. In *Dynamic NMR Spectroscopy*; Academic Press: London, 1982; Chapter 2.
(18) Saturation transfer data as reported in ref 16 also predict a rate of exchange slower than 5 s^{-1} at pH 9.2. Such a slow rate introduces essentially no broadening[17] of the individual 5-methyl lines whose widths in the pure solvents are ~35 Hz.
(19) To ascertain that the effect is not due to His 97 (FG3) or Arg 45 (CD3), which are H bonded to the 7- and 6-heme propionates, respectively (Takano, T. *J. Mol. Biol.* **1977**, *110*, 537–568; 569–584), we used 6-(2-carboxyethyl)-1,3,5,7,8-pentamethyl-2,4-divinylhemin-reconstituted metMbCN (La Mar, G. N.; Emerson, S. D.; Lecomte, J. T. J.; Pande, V.; Smith, K. M.; Craig, G. W.; Kehres, L. A. *J. Am. Chem. Soc.* **1986**, *108*, 5568–5573) (no H bond to His 97) and horse metMbCN (Ar 45 → Lys 45, Dayhoff, M. O. *Atlas of Protein Sequence and Structure*; National Biomedical Research Foundation: Washington, DC 1972; Vol. 5). At pH 9.2, the splitting of the heme methyl line is, in both cases, identical with that of native sperm whale metMbCN.
(20) Norvel, J. C.; Nunes, A. C.; Schoenborn, B. P. *Science (Washington, D. C.)* **1975**, *190*, 568–570.
(21) (a) Shulman, R. G.; Glarum, S. H.; Karplus, M. *J. Mol. Biol.* **1971**, *57*, 93–115. (b) Traylor, T. G.; Berzinis, A. P. *J. Am. Chem. Soc.* **1980**, *102*, 2844–2846.

Structures of the New Binary Metal Carbonyl $Os_4(CO)_{15}$ and $(\eta^5\text{-}C_5Me_5)(OC)IrOs_3(CO)_{11}$. Clusters with Three-Center–Two-Electron Metal–Metal Bonds?

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Of the transition metals, osmium forms the most neutral binary carbonyls. The crystal structures of $Os_3(CO)_{12}$,[1] $Os_5(CO)_{16}$,[2] $Os_5(CO)_{19}$,[3] $Os_6(CO)_{18}$,[4] $Os_7(CO)_{21}$,[5] and $Os_8(CO)_{23}$[6] have been determined. Pentacarbonylosmium, $Os(CO)_5$,[7] and nonacarbonyldiosmium, $Os_2(CO)_9$,[8] are also known as is $Os_6(CO)_{21}$, previously considered to be $Os_6(CO)_{20}$.[3,9] However, to our knowledge there are no previous reports of a tetranuclear binary

(1) Churchill, M. R.; DeBoer, B. G. *Inorg. Chem.* **1977**, *16*, 878.
(2) Eady, C. R.; Johnson, B. F. G.; Lewis, J.; Reichert, B. E.; Sheldrick, G. M. *J. Chem. Soc., Chem. Commun.* **1976**, 271.
(3) Farrar, D. H.; Johnson, B. F. G.; Lewis, J.; Raithby, P. R.; Rosales, M. J. *J. Chem. Soc., Dalton Trans.* **1982**, 2051.
(4) Mason, R.; Thomas, K. M.; Mingos, D. M. P. *J. Am. Chem. Soc.* **1973**, *95*, 3802.
(5) Eady, C. R.; Johnson, B. F. G.; Lewis, J.; Mason, R.; Hitchcock, P. B.; Thomas, K. M. *J. Chem. Soc., Chem. Commun.* **1977**, 385.
(6) Jackson, P. F.; Johnson, B. F. G.; Lewis, J.; Nelson, W. J. H.; Pearsall, M. A.; Raithby, P. R.; Vargas, M. D. *J. Chem. Soc., Dalton Trans.*, in press. See: Vargas, M. D.; Nicholls, J. N. *Adv. Inorg. Chem. Radiochem.* **1986**, *30*, 123.
(7) (a) Calderazzo, F.; L'Eplattenier, F. *Inorg. Chem.* **1967**, *6*, 1220. (b) Rushman, P.; van Buuren, G. N.; Shiralian, M.; Pomeroy, R. K. *Organometallics* **1983**, *2*, 693.
(8) Moss, J. R.; Graham, W. A. G. *J. Chem. Soc., Dalton Trans.* **1977**, 95.
(9) Goudsmit, R. J.; Jeffrey, J. G.; Johnson, B. F. G.; Lewis, J.; McQueen, R. C. S.; Sanders, A. J.; Liu, J.-C. *J. Chem. Soc., Chem. Commun.* **1986**, 24.

0002-7863/87/1509-7220$01.50/0 © 1987 American Chemical Society