Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 142-148

COMPARISON OF THE HEME ELECTRONIC AND MOLECULAR STRUCTURE OF SOYBEAN LEGHEMOGLOBIN AND SPERM WHALE MYOGLOBIN BY PROTON NMR

Gerd N. La Mar, Stephen B. Kong, Kevin M. Smith and Kevin C. Langry

Department of Chemistry, University of California,
Davis, California, 95616.

Received July 27,1981

SUMMARY

The proton nuclear magnetic resonance spectra of soybean ferric leghemoglobin a in the low-spin cyanide and nicotinate complexes have been assigned by specific deuteration of heme methyl groups. The assignments differ from those obtained solely from nuclear Overhauser enhancement measurements and are indicative of a proximal histidyl imidazole-hemin interaction which is very similar to that found in sperm whale myoglobin. The absence of a hyperfine shifted exchangeable NH peak for the distal histidine in leghemoglobin suggests either a very different orientation for this distal ligand or a significantly faster exchange rate with bulk solvent than found in myoglobin.

One of the more interesting monomeric oxygen-binding hemoproteins is the hemoglobin (leghemoglobin) found in the Rhizobium-infected nitrogen fixing root nodules of legumes (1). Although a crystal structure (2) of the lupin leghemoglobin, Lb, acetate complex reveals a tertiary structure very similar to that of myoglobin (3), leghemoglobins exhibit much higher oxygen affinities, due primarily to a much larger O_2 on-rate (4,5), and can readily bind bulky ligands such as nicotinate (6), alkyl isocyanides (7) and long chain carboxylic acids (8). While the X-ray data (2) are in accord with a larger heme pocket than in other oxygen binding hemoproteins, they do not provide any insight into the nature of the protein-heme interactions which are responsible for the rapid O_2 binding.

Proton NMR studies of paramagnetic forms of hemoproteins, particularly in the low-spin ferric state, can provide a wealth of structural information, and

This research was supported by grants from the National Institutes of Health, HL-16087, HL-22252, and the UCD NMR Facility.

0006-291X/81/170142-07$01/00/0

the nature of the hyperfine shifts for heme and axial histidine protons can yield details on the iron-porphyrin and iron-imidazole bonding as modulated by specific protein chains (9-15). Specifically, the pattern of the unambiguously assigned heme methyl resonances can yield direct information on the orientation of the axial imidazole relative to the heme plane (10-13), while the hyperfine shifts for the exchangeable protons for the proximal and distal histidyl imidazoles reflect the strength of the proximal histidine-iron bond and the degree of interaction between coordinated ligand and distal residues (14). Thus proton NMR is well suited for probing the degree of differences and similarities of specific structural features in metMbCN and metLbCN.

Unambiguous assignments of heme resonances of various forms of sperm whale myoglobin based on reconstitution with isotope labeled hemins have been published (11-13,15,16). Recently, a number of the heme resonances of several low-spin Lb proteins, among them the cyanide, metLbCN, and nicotinate, metLbNic, complexes have been reported based on the interpretation of a series of nuclear Overhauser enhancement, NOE, measurements (17,18). The magnitudes and the apparently contrasting pattern of the individual heme methyl hyperfine shifts, as compared to metMbCN (11,15), were interpreted in terms of significant differences in the heme-protein interactions (18). We demonstrate here that parallel studies on these proteins using isotope labeling techniques lead to alternate but unambiguous assignments which suggest a much stronger resemblance in the electronic/molecular structure of the heme pockets of these two proteins.

MATERIALS AND METHODS

The ferric form of legume hemoglobin a was extracted from soybean root nodules and purified as described by Appleby et al. (19). The apo-protein was prepared by extracting the hemin from a cold (0°C) solution of the ferric protein at pH 3.5. The aqueous phase was dialyzed exhaustively against H_2O at 4°C and against 0.15 M Tris HCl, pH 7.35. The apo-protein was reconstituted with [1,3-(C^2H_3)$_2$]-hemin, [1,5-(C^2D_3)$_2$]-hemin and [2,4-(α-C^2H)$_2$]-hemin (16) by adding dropwise a stoichiometric amount of the labeled hemin dissolved in a minimum of 0.01 M NaOH at 0°C to the cold apo-protein solution. The pH was not permitted to exceed 7.4 and the solution stirred for 3 hours. The solution was again dialyzed against H_2O, centrifuged, and either concentrated for H_2O solutions by ultrafiltration, or lyophilized and dissolved in H_2O. Sperm whale myoglobin was purchased from Sigma as a salt-free, lyophilized powder.

NMR samples were prepared 1-3 mM in protein in either 90% H_2O/10% ^2H_2O or 99.9% ^2H_2O 0.2 M in NaCl. The cyanide or nicotinate complexes were prepared by adding a three-fold excess of KCN or nicotinic acid to the solution. The pH was controlled by addition of HCl or NaOH in the appropriate solvent, and measured using an Ingold micro-combination electrode and a Beckman 3550 pH meter; the readings in ^2H_2O are uncorrected for isotope effects and designated 'pH'.

Proton NMR spectra were recorded at 360 MHz on a Nicolet NT-360 spectrometer operating in the quadrature mode. Spectra consisted of approx. 2000 transients collected over a 10 KHz bandwidth using 8K points (10 μs 90° pulse). Signal-to-noise was improved in all spectra by apodization which introduced 3 Hz line broadening. The strong solvent resonance in H_2O solution was suppressed by a 30 ms phase-alternated presaturation pulse. Chemical shifts are referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, and are given in parts per million, ppm, with downfield shifts taken as positive. Both metLbCN and metLbNic in ^2H_2O exhibited proton NMR spectra with shifts identical to those reported earlier at 270 MHz (17,18).

RESULTS

The hyperfine shifted portions of the 360 MHz proton NMR spectrum of sperm whale metMbCN in H_2O (A) is compared with those of metLbCN in H_2O (B) and ^2H_2O (C) of Figure 1. The previous assignments for the proximal and distal histidyl imidazole NH peaks from T_1 measurements (14) and for the heme resonances based on deuterium labeling (15) are included for metMbCN. The two heme methyl signals a and b in metLbCN are apparent, as reported earlier at 270 MHz (17,18), and comparison of the H_2O and ^2H_2O spectra of metLbCN reveals a single strongly hyperfine shifted proton peak, y, which T_1 measurements and pH influences on lability dictate (14,20) must arise from the proximal histidyl imidazole NH.

The assignments of methyl and vinyl peaks via deuterium labeling are illustrated in Figure 2 for metLbNic in ^2H_2O. All previously reported NOE effects for this complex could be duplicated quantitatively (17,18). However, while peak d is confirmed as a vinyl-H_α, methyl peaks a, b and c clearly arise from 5-CH_3, 1-CH_3 and 3-CH_3, in contrast to the previous NOE-based assignment of a and b to 8-CH_3 and 3-CH_3 (18). The results of similar isotope labeling (20) of metLbCN (not shown) are included in B of Figure 1 and confirm the same discrepancy with the earlier published results (18).

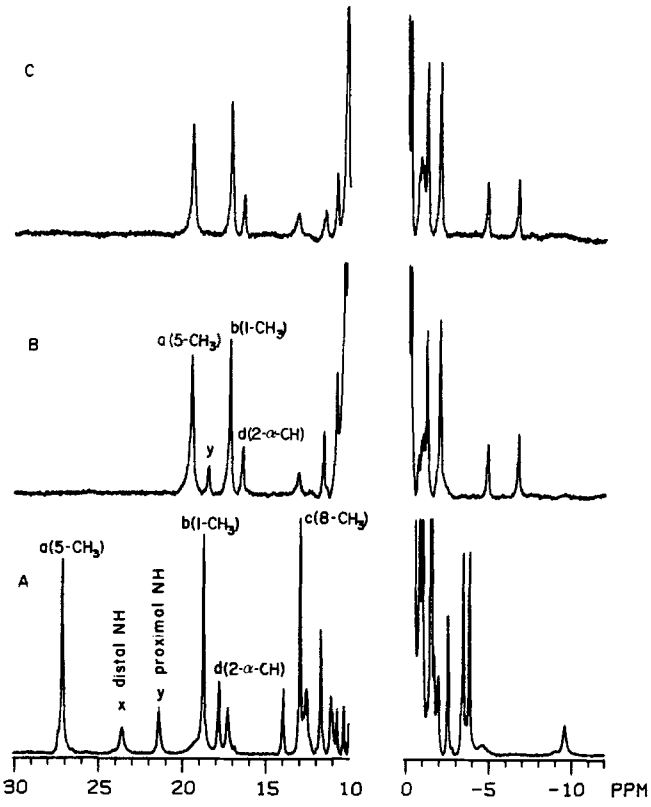

Figure 1: Hyperfine-shifted portions of the 360 MHz proton NMR spectra of: A. sperm whale metMbCN, 25°C, pH 8.4 in 90% H_2O/10% ^2H_2O, with the assignments from Refs. 14, 15 and 25; B. soybean metLbCN, 25°C, pH 8.3 in 90% H_2O/10% ^2H_2O, with assignments from Ref. 20; C. soybean metLbCN, 25°C, 'pH' 8.6 in ^2H_2O. Shifts are in ppm from DSS.

DISCUSSION

The present unambiguous heme methyl assignments differ from the previous NOE-based assignments by a 180° rotation about the α-γ-meso axis. The origin of the discrepancy is that NOE measurements of an insufficient number of resonances were made which necessitated the assumption of one assignment based on arguments (18) related to the crystal structure of lupin Lb (2). Unfortunately the comparison was invalid. That NOE measurements alone can establish unique assignments if a large enough number of peaks are resolved is illustrated in the case of ferricytochrome b_5 where the NOE-based (21) and isotope labeling (13) assignments coincided. In general, a single isotope labeling appears necessary in order to be able to place confidence in the remainder of

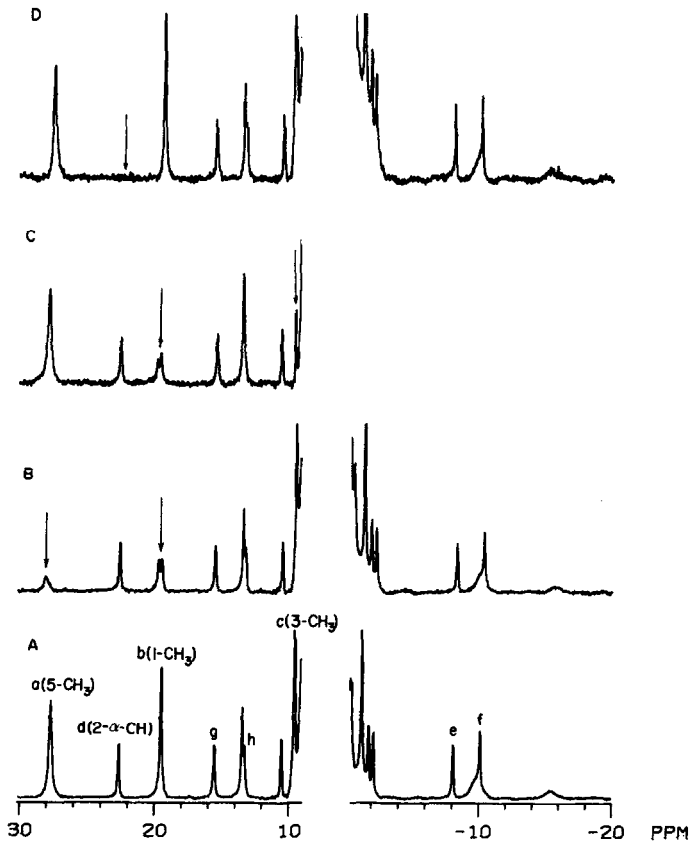

Figure 2: Hyperfine-shifted portions of the met nicotinate complexes of leghemoglobin a at 25°C, in ^2H_2O at 'pH' ~6.6; A, native protein; and metLbNic reconstituted with: B, [1,5-$(C^2H_3)_2$]-hemin; C, [1,3-$(C^2H_3)_2$]-hemin; D, [2,4-$(\alpha\text{-}CH)_2$]-hemin. Vertical arrows indicate reduced signal intensities due to deuteration. Shifts are in ppm from DSS.

the NOE-based assignments. Since the previously reported (17,18) NOE effects could be quantitatively duplicated, d, e, f (Fig. 2 A) can be assigned to the 2 rather than the 4-vinyl group and g, h to the 6- rather than the 7-α-CH_2 (18).

Comparison of the spectra of correctly assigned metMbCN and metLbCN now reflect very similar rhombic distortions which are indicative of essentially the same orientation of the proximal histidyl imidazole relative to the heme (10-13,15). While we confirm that the hyperfine shifts for the heme methyl signals are smaller in metLbCN than in metMbCN, we cannot simply interpret this as indicating that the axial interaction must be stronger, as suggested earlier (18), since the pyrrole 2,4-H of deuterohemin-reconstituted into metLbCN (not

shown) exhibits larger upfield hyperfine shifts (20) than in the analogous reconstituted metMbCN (22). Thus the smaller downfield methyl shifts in metLbCN are indicative of an overall upfield bias for all shifts which is suggestive (23,24) of an increase in axial magnet anisotropy rather than a decrease in iron-porphyrin covalency.

The hyperfine shifts for the proximal histidyl imidazole NH's (peak y) are similar in metLbCN and metMbCN (14,25). If the magnetic anisotropy is indeed larger in metLbCN, then the NH contact shift in that protein is smaller (23), which can be interpreted either in terms of weaker Fe-imidazole bonding, as suggested in the crystal structure (2), or stronger NH hydrogen bonding (26) to some acceptor in metLbCN; however, both reflect properties of the ligated state which would influence primarily the ligand off-rate. The strongest contrast in the proton NMR spectra of the two proteins is the absence of a strongly hyperfine shifted signal assignable to the distal histidyl imidazole NH in metLbCN (14,25). This requires that either the distal ligand in Lb is further removed from the iron than in metMbCN, which could decrease steric interference with ligation, or that the larger heme pocket in metLbCN permits rapid exchange of the distal histidyl imidazole NH with bulk water. A greater heme accessibility in Lb for other substrates has already been established (27). Such enhanced accessibility of ligands may be related to the greater O_2 on-rate (4,5). Detailed studies of the dynamic stability of the heme pocket of various forms of Lb, as determined by hydrogen exchange kinetics of assigned resonances in the heme cavity, are in progress, and their comparison to similar data recently reported on identical forms of myoglobin (14,28) may further shed light on the physical basis for the contrasting ligand on-rates in these proteins.

REFERENCES

1. Appleby, C.A. (1974): The Biology of Nitrogen Fixation (Quispel, A., ed.) pp 521-534, North-Holland, Amsterdam.
2. Arutyunyan, E.G., Kuranova, I.P., Vainshtein, B. and Steigemann, W. (1980) Sov. Phys. Crystallogr. 25, 43-58.

3. Takano, T. (1977) J. Mol. Biol. 110, 537-586.
4. Wittenberg, J., Appleby, C.A. and Wittenberg, B.A. (1972) J. Biol. Chem. 247, 527-531.
5. Imamura, T., Riggs, A. and Gibson, Q.H. (1972) J. Biol. Chem. 247, 521-526.
6. Appleby, C.A., Wittenberg, B.A. and Wittenberg, J.B. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 514-568.
7. Stetzkowski, E., Banerjee, R., Lallemand, J.-Y., Cendrier, B. and Mansuy, D. (1980) Biochemie 62, 795-801.
8. Ellfolk, N. (1961) Acta Chem. Scand. 15, 915-984.
9. Morrow, J.S. and Gurd, F.R.N. (1975) CRC Crit. Rev. Biochem. 3, 221-287.
10. La Mar, G.N. (1979): Biological Applications of Magnetic Resonance (Shulman, R.G., ed.) pp 305-343, Academic Press, New York.
11. La Mar, G.N., Budd, D.L., Viscio, D.L., Smith, K.M. and Langry, K.C. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5755-5759.
12. La Mar, G.N., Smith, K.M., Gersonde, K., Sick, H. and Overkamp, M. (1980) J. Biol. Chem. 255, 66-70.
13. La Mar, G.N., Burns, P.D., Jackson, J.T., Smith, K.M., Langry, K.C. and Strittmatter, P. (1981) J. Biol. Chem. 256, 6075-6079.
14. Cutnell, J.D., La Mar, G.N. and Kong, S.B. (1981) J. Am. Chem. Soc. 103, 3567-3572.
15. Mayer, A., Ogawa, S., Shulman, R.G., Yamane, T., Cavaleiro, J.A.S., Rocha-Gonsalves, A.M.d'A., Kenner, G.W. and Smith, K.M. (1974) J. Mol. Biol. 86, 749-756.
16. La Mar, G.N., Budd, D.L., Smith, K.M. and Langry, K.C. (1980) J. Am. Chem. Soc. 102, 1822-1827.
17. Trewhella, J., Wright, P.E. and Appleby, C.A. (1979) Nature 280, 87-88.
18. Trewhalla, J. and Wright, P.E. (1980) Biochim. Biophys. Acta 625, 202-220.
19. Appleby, C.A., Nicola, N.A., Hurrell, J.G.R. and Leach, S.J. (1975) Biochemistry 14, 4444-4450.
20. Kong, S.B. (1981) Ph.D. Thesis, University of California, Davis.
21. Keller, R.M. and Wüthrich, K. (1980) Biochim. Biophys. Acta 621, 204-217.
22. Shulman, R.G., Wüthrich, K., Yamane, T., Antonini, E. and Brunori, M. (1969) Proc. Natl. Acad. Sci. U.S.A. 63, 623-628.
23. La Mar, G.N. and Walker, F.A. (1978): The Porphyrins (Dolphin, D., ed.) Vol IV, pp 61-157, Academic Press, New York.
24. La Mar, G.N., Del Gaudio, J. and Frye, J.S. (1977) Biochim. Biophys. Acta 458, 522-435.
25. Sheard, B., Yamane, T. and Shulman, R.G. (1970) J. Mol. Biol. 53, 35-48.
26. Valentine, J.S., Sheridan, R.P., Allen, L.C. and Kahn, P.C. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 1009-1013.
27. Ollis, D.L., Wright, P.E., Pope, J.M. and Appleby, C.A. (1981) Biochemistry 20, 587-594.
28. La Mar, G.N., Cutnell, J.D. and Kong, S.B. (1981) Biophys. J. 84, 217-226.