HIGH RESOLUTION NMR STUDIES OF SOYBEAN LEGHEMOGLOBIN *a*

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1. Introduction

Leghemoglobins (Lbs) are monomeric hemeproteins containing one heme group per molecule and are found in *Rhizobium*-infected nitrogen-fixing legume root nodules. They appear to function by facilitating the diffusion of oxygen to the nitrogen-fixing bacteroids at stabilized low oxygen tension [1]. Leghemoglobins qualitatively resemble vertebrate myoglobins (Mbs) in their optical spectra and oxygen- and ligand-binding properties. Interest in the structure of leghemoglobin primarily arises from its very high oxygen affinity and extremely rapid oxygen 'on' reaction [2], from its ability to accept bulky ligands such as nicotinic acid [3] and long chain carboxylic acids [4] and from the tendency of the ferric protein to exist as an equilibrium mixture of spin states [5,6].

In view of the considerable success of NMR spectroscopy in probing the structures of hemeproteins [7,8] we have undertaken a detailed NMR study of leghemoglobin. The present paper presents some preliminary results for soybean leghemoglobin *a* (Lb*a*) and its cyanide, oxygen and carbonmonoxide complexes.

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2. Materials and methods

Ferric leghemoglobin *a* was extracted from soybean root nodules and purified by a previously described procedure [9]. Solutions for NMR were approx. 5 mM in Lb. The solvent was exchanged by repeated dialysis against D_2O and the pH adjusted to 7.0 by careful addition of DCl or NaOD. Leghemoglobin cyanide (LbCN) was prepared by addition of a 5-fold excess of KCN in D_2O solution to ferric Lb and the pH was readjusted to 7.0. Oxyleghemoglobin (LbO_2) was prepared by reduction of nitrogen-flushed ferric Lb with a 2-fold excess of sodium dithionite followed by passage through a small column of Sephadex G15 containing air-equilibrated 10 mM phosphate, 0.1 mM EDTA buffer (pH 7) in D_2O. Carbonmonoxy-leghemoglobin (LbCO) was isolated from a similar column containing CO equilibrated buffer. The optical spectra of all the complexes were in close agreement with published spectra [1]. Spectra of sperm whale myoglobin (Koch-Light Laboratories) in D_2O at pH 7.0 were recorded for comparison with those of Lb.

NMR spectra were recorded using a Bruker 270 MHz spectrometer fitted with an Oxford Instrument Company magnet and operating in the pulsed Fourier transform mode. Dioxane was used as an internal standard but all peaks are referred to TSS.

3. Results and discussion

The spectrum of ferric leghemoglobin is shown in fig.1(a). The resolution may be enhanced by application of the convolution difference technique [10], several peaks then showing evidence of spin–spin coupling. Many peaks are resolved outside the central overlapping region of the protein spectrum (approx. 9–0.5 ppm). These arise from protons near to aromatic amino acids or to the predominantly high spin ferric heme and are thus subject to ring current and/or paramagnetic shifts. The positions of these peaks are very sensitive to the structure of the protein. One notable feature of the spectrum in the aromatic region is a sharp resonance of intensity one proton at 7.14 ppm. Application of the Carr Purcell method A pulse sequence to determine multiplet structure [11] indicates that this resonance is a singlet. It must therefore arise from the C-2 proton of tryptophan or from the C-2 or C-4 protons of histidine. As both histidine residues in Lb *a* have been implicated near the heme (as an iron ligand and distal group respectively) [6] and are thus expected to give rise to greatly broadened resonances, the 7.14 ppm singlet resonance is likely to arise from the C-2 proton of a tryptophan residue distant from the heme. A sharp resonance of intensity approx. one proton also occurs at 7.14 ppm in the spectra of LbCN, LbCO and LbO_2. This insensitivity to changes in the iron spin state or oxidation state is clearly consistent with the above assignment.

In the low spin cyanide derivative of ferric leghemoglobin the paramagnetic line broadening produced by the high-spin ferric heme is considerably reduced and resonances of porphyrin ring protons and the iron ligands may be clearly resolved (fig.2). These are often shifted outside the main protein envelope by contact and pseudocontact interactions with the unpaired iron electron spin. Thus resonances at 19.5 ppm and 17.2 ppm, each of intensity three protons (measured relative to TSS as an internal intensity standard under conditions which avoid saturation of the TSS peak) in the spectrum of LbCN at 25°C are assigned to two of the four porphyrin ring methyl groups on the basis of their intensities and previous studies of the NMR

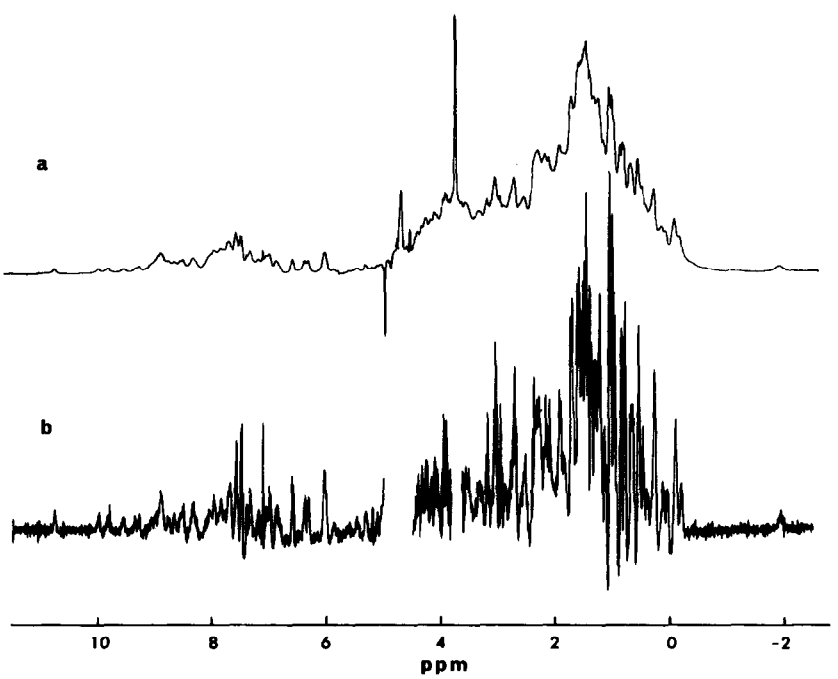

Fig.1. NMR spectrum of ferric soybean leghemoglobin *a* at 25°C. (a) Normal FT spectrum. (b) Convolution difference spectrum. In this and the following spectra the residual solvent peak has been suppressed by selective irradiation and the peak at 3.75 ppm is from the dioxane reference.

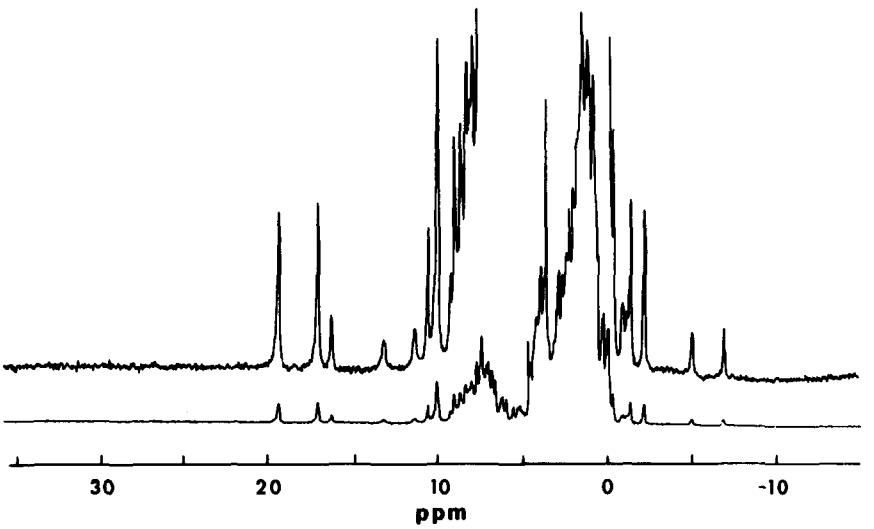

Fig.2. NMR spectrum of ferric leghemoglobin cyanide complex at 25°C.

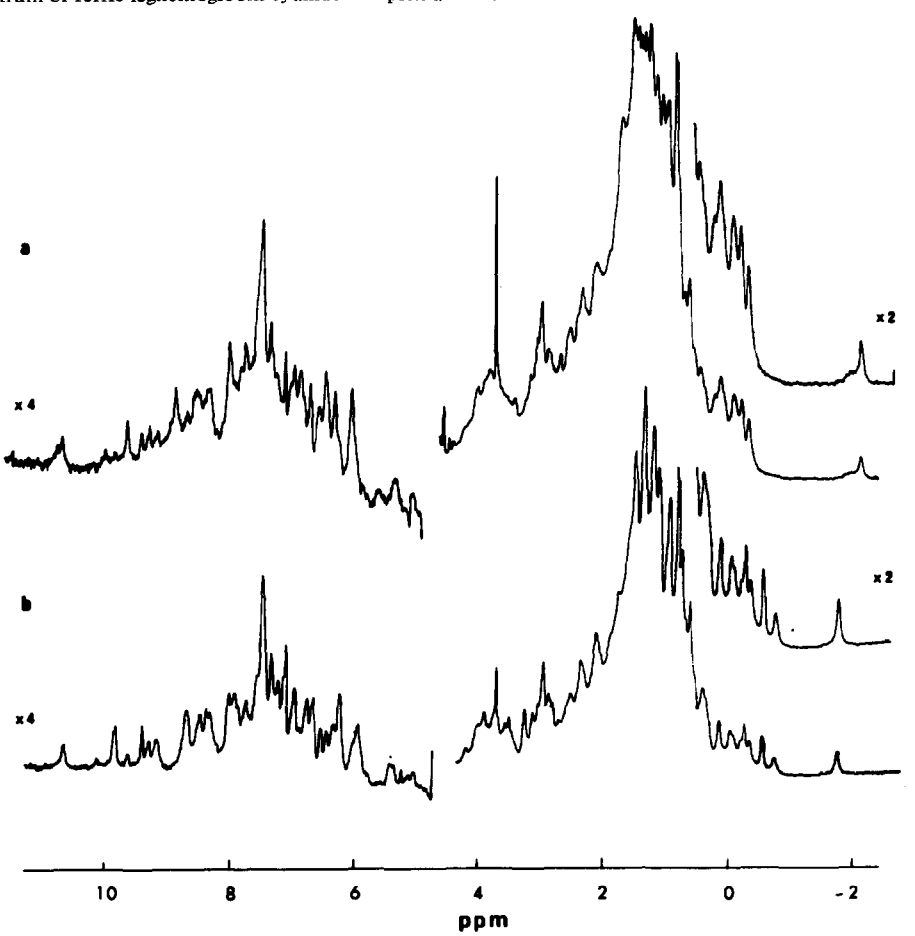

Fig.3. NMR spectra at 25°C of (a) ferrous oxyleghemoglobin, and (b) ferrous carbonmonoxyleghemoglobin.

spectra of hemeproteins and free hemin complexes [7]. The remaining two heme methyl-group proton resonances have not yet been identified but presumably resonate at higher fields where they may be hidden by other overlapping resonances. It is of interest that the distribution of porphyrin ring methyl-group proton resonances in LbCN is very different from that in MbCN (with heme methyl-group resonances at 27 ppm, 19 ppm and 13 ppm) [12]. This is clearly indicative of differences in the heme electronic structures of the cyanide complexes of leghemoglobin and myoglobin.

Comparison of the spectra of the diamagnetic oxygen and carbon monoxide complexes of ferrous leghemoglobin (fig.3) reveal many differences, particularly in the low field region from 10–6 ppm where aromatic side-chain protons and non-exchanged NH protons resonate, and at fields higher than 2 ppm where peaks due to aliphatic CH_2 and CH_3 protons usually occur. These differences are indicative of some structural rearrangement between the O_2 and CO complexes. Of particular interest are the resonances at higher fields than 0.5 ppm since these are subject to upfield ring current shifts from aromatic amino acids or the porphyrin ring and, as such, are highly sensitive to the protein conformation. Again, differences are apparent between LbO_2 and LbCO. In particular, the chemical shift of the resonance at highest field varies by 0.4 ppm between LbO_2 and LbCO. This resonance must be subject to a ring current shift of the order of 3 ppm or greater in LbO_2. A ring current shift of this magnitude is most likely caused by the porphyrin ring rather than aromatic amino acids. The observed sensitivity of this resonance to the nature of the axial ligands and to the spin state and oxidation state of the iron atom further suggests that it arises from a side-chain close to the heme. Estimates of ring current shifts associated with porphyrin rings [13] indicate a movement of several tenths of an Angstrom for this residue to account for the difference in its chemical shift between LbO_2 and LbCO. Similarly resonances at –0.55 ppm and –0.75 ppm in the spectrum of LbCO must be shifted downfield by at least 0.2 ppm and 0.4 ppm, respectively, in the spectrum of LbO_2. Clearly there is some significant difference in structure between oxy- and carbonmonoxy-leghemoglobin, suggesting some degree of protein conformational flexibility near the heme.

It is of particular interest to compare the NMR spectrum of ferric leghemoglobin with that of metmyoglobin to look for evidence of conformational differences between them. Most notable in the spectrum of ferric leghemoglobin is the relatively small number of peaks at fields higher than 0 ppm (six resonances) relative to the ferric myoglobin spectrum in the same region (twelve resonances) (fig.4). These resonances arise from CH_2 and CH_3 protons of aliphatic amino acids shifted to high fields by ring current and/or pseudocontact interactions. (Contact shifted ligand resonances are expected to be too broad to be detected in this region [8].) That most of these resonances are subject to pseudocontact shifts and thus arise from amino acids near the heme is evident from their sensitivity to added acetate (which forms a purely high spin complex with ferric Lb [6]) and from their temperature-dependent chemical shifts (P. E. Wright and C. A. Appleby, unpublished observations). In the case of high spin ferric hemes and hemeproteins, an upfield pseudocontact shift occurs for resonances of protons located above or below the heme plane and within the pseudocontact shift cone [8]. The simplest explanations

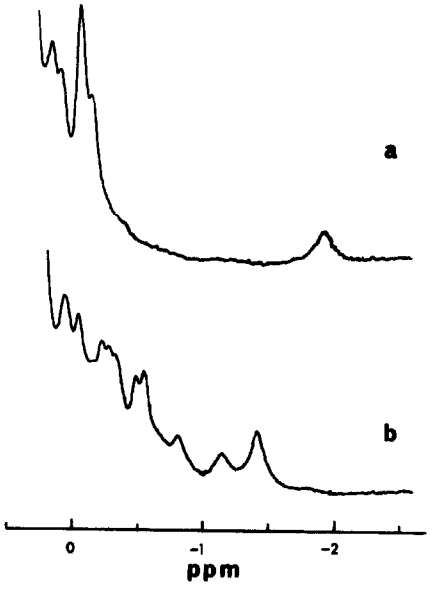

Fig.4. Part of the high field region of the NMR spectra at 25°C of (a) ferric leghemoglobin, and (b) ferric metmyoglobin. Further resonances (not shown) occur at –3.0, –3.7 and –6.7 ppm for leghemoglobin and –4.1, –5.2 and –7.3 ppm for myoglobin.

for the fewer resonances subject to upfield pseudocontact shifts in Lb as compared to Mb is that the heme pocket is more open in the former with fewer amino acid side-chains making close approaches to the heme from above the plane of the porphyrin ring. Similar conclusions have been reached from circular dichroism measurements [14]. An alternative explanation is that upfield pseudocontact shifts are smaller in magnitude in ferric Lb than in metmyoglobin due to exchange averaging of the shift due to high spin and low spin components. However this seems unlikely since addition of acetate causes no detectable upfield shift of the high field temperature dependent resonances as would then be expected upon formation of a fully high spin ferric complex.

The spectrum of ferric metmyoglobin recorded over sufficiently large sweep widths reveals resonances of porphyrin ring protons at very low fields which are subject to very large paramagnetic shifts due to the $S = 5/2$ iron atom. Thus for Mb at 36°C, several resonances are detected with widths of the order of 150 Hz to fields as low as 88 ppm (fig.5). This is in marked contrast to the spectrum of ferric Lb over the same region in which only two very broad resonances (line widths greater than 800 Hz) can be resolved at temperatures up to 36°C and the remaining porphyrin peaks are clearly broadened beyond detection. Two explanations for the broadness of the porphyrin ring resonances in Lb are apparent. Firstly broad peaks may arise from a considerably longer unpaired electron spin relaxation time in Lb than in Mb. However, in view of the similarities of the magnetic and optical properties of the two heme proteins [1], this is considered unlikely. A more tenable explanation invokes

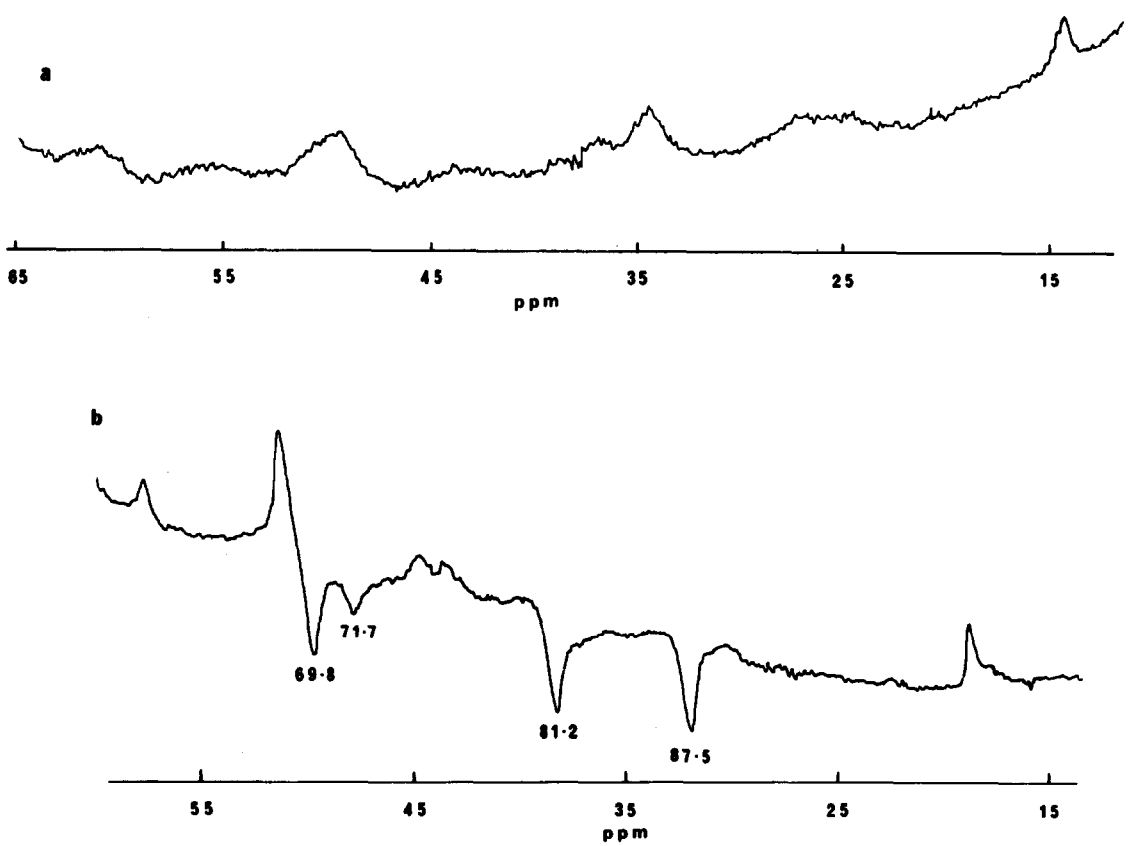

Fig.5. (a) Low field region of the NMR spectrum (width 20 kHz) of ferric leghemoglobin at 36°C. (b) Low field region of the NMR spectrum (width 20 kHz) of ferric metmyoglobin at 36°C. The inverted resonances are of lower frequency than the central pulse frequency and are 'folded back' into the spectrum. Their chemical shifts are indicated on the diagram.

exchange between high and low spin states in ferric leghemoglobin at such a rate that exchange broadening of the porphyrin resonances occurs. Since the rate of spin exchange must be comparable to the difference in chemical shift of porphyrin resonances in the high spin and low spin forms, an estimate of the rate of exchange can be obtained. Thus for a porphyrin ring proton resonating at approx. 90 ppm in the high spin state and at approx. 20 ppm in the low spin state (cf. LbCN), exchange broadening would occur at a spin state interconversion rate of approx. $10^5 s^{-1}$. This rate, which is in excellent agreement with the value obtained by *T*-jump measurements [15], is slower than is usual for hemeproteins [16] and suggests that protein conformational changes are involved in the spin equilibrium. A low spin form of ferric Lb in which the axial iron ligands are both histidine residues is observed in the low temperature ESR spectrum [6]. It is therefore likely that the spin state equilibrium at higher temperature arises through flexibility of the protein close to the heme, enabling the distal histidine side-chain to move on and off the iron atom.

4. Conclusions

The present NMR experiments have shown the heme pocket in ferric soybean leghemoglobin to be a relatively open, flexible region of the protein. An open heme pocket would clearly facilitate rapid oxygen binding. Subsequent conformational changes to close the heme pocket could then stabilise the resulting oxygen complex.

Acknowledgements

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References

[1] Appleby, C. A. (1974) in: The Biology of Nitrogen Fixation (Quispel, A. ed) pp. 521–554, North-Holland Publishing Co., Amsterdam.
[2] Wittenberg, J. B., Appleby, C. A. and Wittenberg, B. A. (1972) J. Biol. Chem. 247, 527–531.
[3] Appleby, C. A., Wittenberg, B. A. and Wittenberg, J. B. (1973) Proc. Natl. Acad. Sci. USA 70, 564–568.
[4] Ellfolk, N. (1961) Acta Chem. Scand. 15, 975–984.
[5] Ehrenberg, A. and Ellfolk, N. (1963) Acta Chem. Scand. 17, S343–S347.
[6] Appleby, C. A., Blumberg, W. E., Peisach, J., Wittenberg, B. A. and Wittenberg, J. B. (1976) J. Biol. Chem. 251, 6090–6096.
[7] Wuthrich, K. (1970) Struct. Bonding 8, 53–121.
[8] Williams, R. J. P., Wright, P. E., Mazza, G. and Ricard, J. R. (1975) Biochim. Biophys. Acta 412, 127–147.
[9] Appleby, C. A., Nicola, N. A., Hurrell, J. G. R. and Leach, S. J. (1975) Biochemistry 14, 4444–4450.
[10] Campbell, I. D., Dobson, C. M., Williams, R. J. P. and Xavier, A. V. (1973) J. Magn. Resonance 11, 172–181.
[11] Campbell, I. D., Dobson, C. M., Williams, R. J. P. and Wright, P. E. (1975) FEBS Lett. 57, 96–99.
[12] 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.
[13] Shulman, R. G., Wutrich, K., Yamane, T., Patel, D. J. and Blumberg, W. E. (1970) J. Mol. Biol. 53, 143–157.
[14] Nicola, N. A., Minasian, E., Appleby, C. A. and Leach, S. J. (1975) Biochemistry 14, 5141–5148.
[15] Dyson, H. J., Beattie, J. K. and Appleby, C. A., to be published.
[16] Beattie, J. K. and West, R. J. (1974) J. Amer. Chem. Soc. 96, 1933–1935.