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SPIN STATE EQUILIBRIA IN SOYBEAN FERRIC LEGHEMOGLOBIN AND ITS COMPLEXES WITH FORMATE AND ACETATE

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SUMMARY

The interactions of fluoride, acetate and formate with soybean ferric leghemoglobin a have been investigated by ^{1}H NMR spectroscopy. In the presence of fluoride or acetate leghemoglobin is locked into a high spin ferric conformation whilst the formate complex exists as an equilibrium mixture of high and low spin states. Both formate and acetate ligate directly to the iron and the different magnetic properties of the complexes are attributed to steric constraints within the heme pocket.

Leghemoglobins are monomeric hemeproteins which function in oxygen transport in Rhizobium-infected, nitrogen-fixing legume root nodules (1). The tertiary structure of leghemoglobin from lupin has been determined to 2.8 Å resolution and is very similar to that of myoglobin (2). Despite this structural resemblance, leghemoglobin differs from myoglobin in many of its properties (1), including its ability to bind long chain carboxylic acids (3) and the tendency of the ferric protein to exist as an equilibrium mixture of spin states (4,5). In order to gain an insight into the molecular basis for these and other properties we have undertaken detailed NMR studies of leghemoglobin (6). We report here on the interaction of soybean ferric leghemoglobin a with fluoride, acetate and formate. We show that acetate and formate are both iron ligands and discuss unexpected differences in the iron spin state in these carboxylate complexes.

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MATERIALS AND METHODS

Soybean ferric leghemoglobin a was purified by a previously described procedure (7). For NMR measurements the protein was repeatedly dialysed against phosphate buffer (10mM, pH 7.0) in D_2O. The carboxylate complexes were prepared by addition of sodium deuteroacetate (28-fold excess) or sodium formate (10-fold excess) to ferric leghemoglobin. The pH was adjusted to 6.5 by careful addition of DCl or NaOD. Proton NMR spectra were recorded using a Bruker HX-270 spectrometer. Dioxan was used as an internal standard but all peaks are referred to TSS (trimethylsilylpropanesulphonic acid). Optical difference spectra were recorded using a Cary 14 spectrophotometer.

RESULTS AND DISCUSSION

The NMR spectrum of ferric leghemoglobin in the low field region is shown in fig. 1(a). The absence of hyperfine-shifted heme methyl group resonances has been reported by us previously (6) and is attributed to broadening due to relatively slow exchange (ca. $10^5 s^{-1}$) between high spin and low spin states at ambient temperatures. Ligation of fluoride to the heme iron atom locks leghemoglobin into a fully high spin ferric configuration (5) and gives rise to a broad set of resonances near 50 ppm (fig. 1(b)) which probably arise from the heme methyl group protons. Their broadness is attributed to the slow iron unpaired electron spin relaxation characteristic of the fluoride complex of leghemoglobin and other hemeproteins (8). A similar spectrum has been reported for myoglobin fluoride (9).

Acetate binds reversibly to ferric leghemoglobin to form a high spin complex (4,5). In the spectrum of leghemoglobin deuteroacetate well-resolved heme methyl group resonances are observed at 65.9, 62.9, 61.0 and 54.9 ppm at 30°C (fig. 1(c)). Clearly deuteroacetate, like fluoride, locks leghemoglobin into a high spin ferric configuration. The heme methyl group protons resonate in the same region as those of the abnormal β-subunits of hemoglobin M Milwaukee (10) in which a carboxylate group is ligated to the iron atom (11). Thus the NMR spectrum clearly indicates direct ligation of deuteroacetate to the iron atom in leghemoglobin. This conclusion has recently been confirmed for the acetate complex of lupin leghemoglobin by

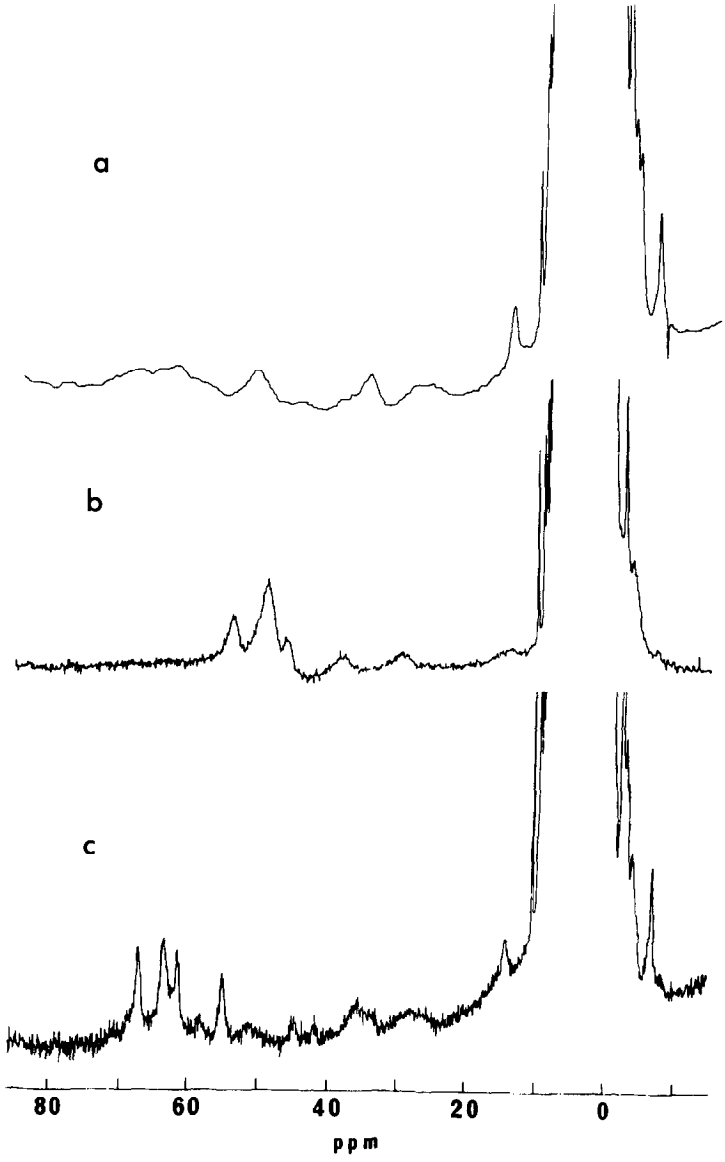


Figure 1. 270 MHz proton NMR spectra at 25°C of (a) ferric soybean leghemoglobin a (2.6 mM, pH 7.0). (b) Leghemoglobin fluoride (42 mM fluoride, pH 6.5). (c) Leghemoglobin deuteroacetate (27 mM deuteroacetate, pH 6.5).

X-ray diffraction (12). The observed temperature dependence of the hyperfine-shifted heme methyl group resonances confirms that leghemoglobin deuteroacetate is in the high spin ferric state (fig. 2). The resonances exhibit a normal Curie law (1/T) temperature dependence and shift upfield

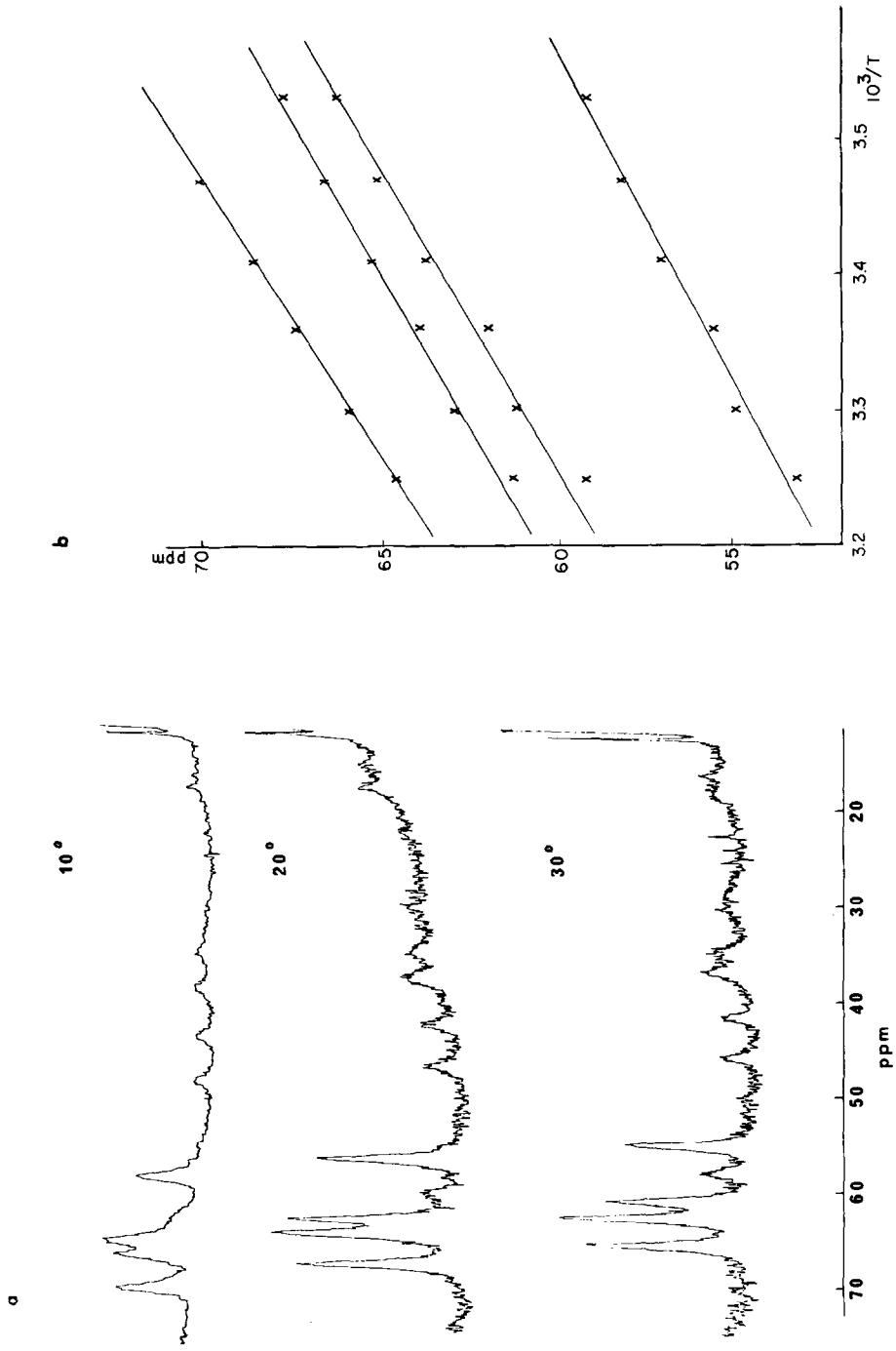


Figure 2. (a) Temperature dependence of the proton NMR spectrum of leghemoglobin deuteroacetate. (b) Plot of chemical shift versus inverse temperature for the heme methyl group proton resonances.

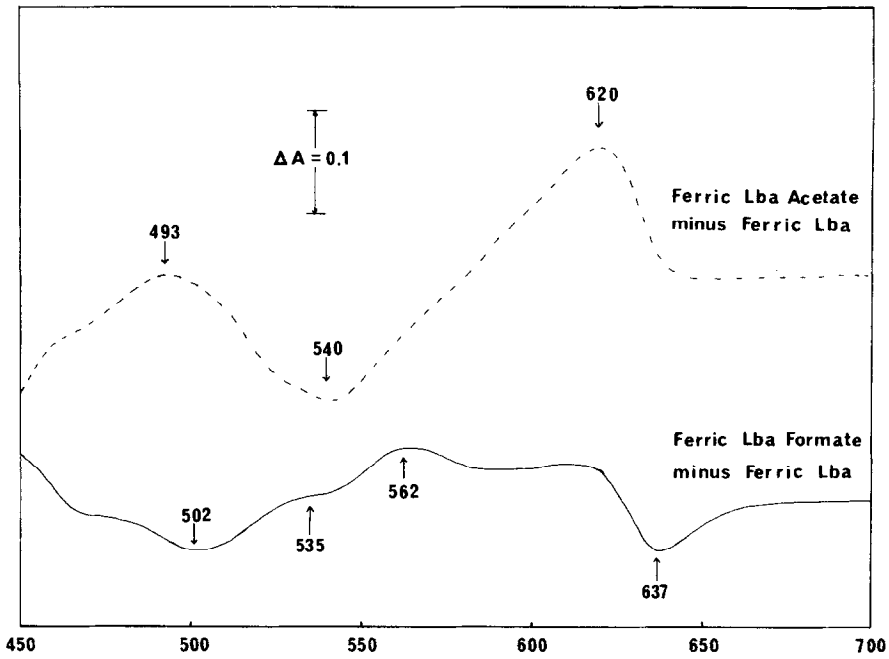
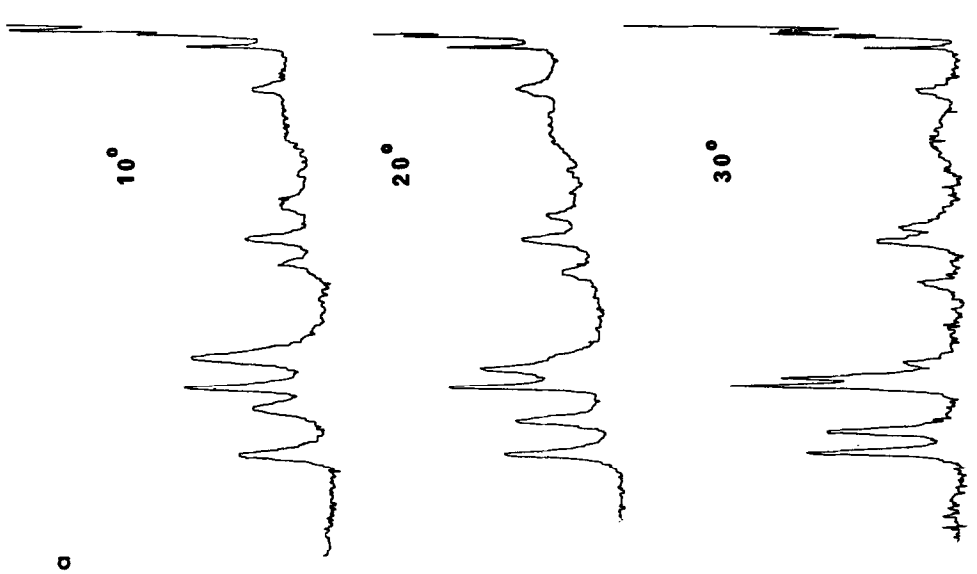


Figure 3. Optical difference spectra for acetate and formate complexes of soybean leghemoglobin (50 μM in 50 mM MES/NaOH buffer, pH 5.30 at 20°C). (a) Spectrum of leghemoglobin acetate (total acetate concentration is 250 mM) versus leghemoglobin. (b) Spectrum of leghemoglobin formate (total formate concentration is 250 mM) versus leghemoglobin.

towards the positions at which they would resonate in a diamagnetic heme-protein (ca. 3 ppm).

It is clear from the optical difference spectra of fig. 3 that formate interacts with leghemoglobin in a rather different manner to acetate (and other straight chain aliphatic carboxylates). The charge transfer band peaks at 620 nm and 493 nm and the ferric hemochrome trough at 540 nm are in accord with formation of a high spin complex when ferric leghemoglobin combines with acetate at 20°C. On reaction with formate, however, the appearance of ferric hemochrome peaks at 535 and 562 nm and charge transfer band troughs at 502 and 637 nm in the difference spectrum suggests that ferric leghemoglobin adopts a more low spin structure.

Further information on the magnetic state of the formate complex of leghemoglobin was obtained from the NMR spectrum which exhibits well-resolved resonances at 45.1, 43.3, 39.3 and 38.6 ppm at 30°C arising from the four heme methyl groups (fig. 4). These are observed at considerably higher fields than the corresponding resonances in the spectrum of leghemoglobin acetate. Further, their temperature dependence does not follow the Curie law since two of them shift downfield with increasing temperature, i.e., move away from their diamagnetic positions (fig. 4). The other two resonances exhibit much smaller temperature dependence than do the corresponding resonances of leghemoglobin acetate. Such behaviour reflects an increase in hyperfine shift with increasing temperature. This is clear indication of the existence of an equilibrium mixture of spin states in leghemoglobin formate, an increase in temperature leading to an increase in the proportion of the high spin species. The temperature dependence of the optical spectrum is in accord with this conclusion. Similar observations have been made for the azide and imidazole complexes of myoglobin (13). Exchange between formate, acetate and ferric leghemoglobin is slow on the NMR time scale. The rate of interconversion between the high spin and low spin states of leghemoglobin formate is fast since the resonances are observed to shift steadily with changing temperature. This is in marked contrast to the relatively slow spin state exchange rate (ca. $10^5 s^{-1}$) observed in ferric leghemoglobin (6) and indicates differences in the origin of the spin state equilibria. The rapid spin state interconversion in leghemoglobin formate probably arises from intersystem crossing between ^6A and ^2T energy states, constrained to lie close in energy by the ligand field produced by formate, the proximal histidine and the four pyrrole nitrogen donor atoms. Such intersystem crossing is generally very rapid (14). We have previously suggested that the slower spin state exchange in ferric leghemoglobin arises from a protein conformational change and probably involves motion of the distal histidine on and off the iron atom (6).



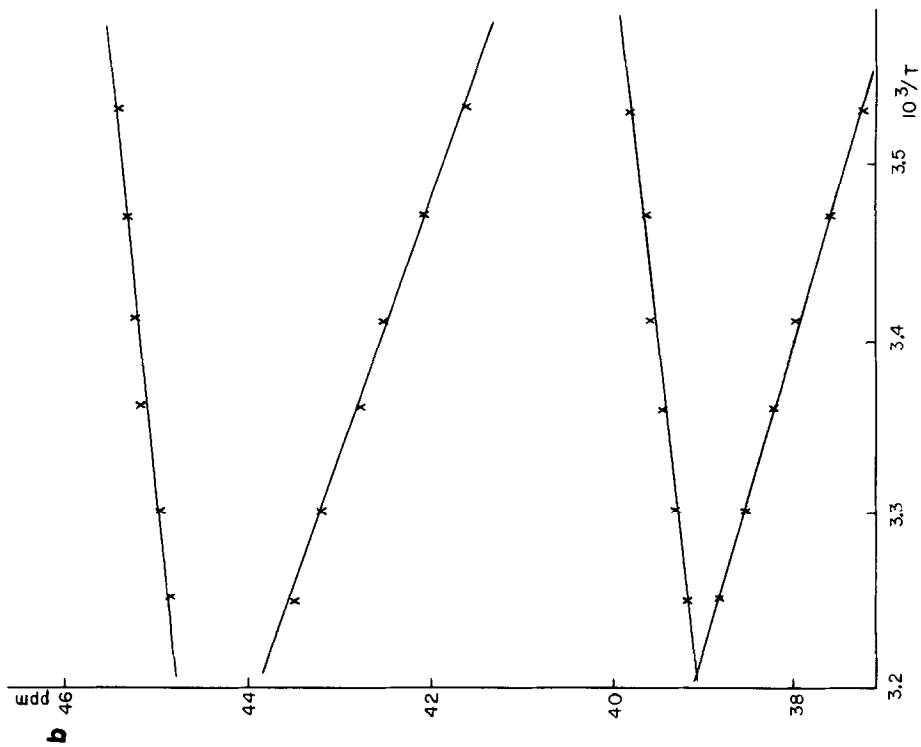


Figure 4. (a) Temperature dependence of the proton NMR spectrum of leghemoglobin formate. (b) Plot of chemical shift versus inverse temperature for the heme methyl group proton resonances.

Why does the formate complex of leghemoglobin exist as an equilibrium mixture of high spin and low spin states at ambient temperatures whilst the acetate complex is fully high spin? This behaviour is unique to leghemoglobin, myoglobin formate for example being a high spin ferric complex with NMR spectrum similar to that of leghemoglobin deuteroacetate (9). The inherent ligand field strengths of formate and acetate ligands are expected to be very similar, in which case their complexes with leghemoglobin should exhibit similar magnetic properties. The observed differences in leghemoglobin formate and leghemoglobin acetate can, however, be rationalised if it is assumed that steric constraints within the heme pocket interfere with the binding of the bulkier acetate ligand, leading to a weaker ligand field and a high spin ferric complex. In support of this interpretation, formate binds more strongly to ferric leghemoglobin (K_{diss} = 0.23 mM, pH 5.3 and 20°C) than does acetate (K_{diss} = 4.5 mM, pH 5.3 and 20°C) (15), which has an affinity for leghemoglobin similar to that of longer straight chain carboxylic acids (3). Substitution at the α-CH_2 group of aliphatic carboxylic acids substantially reduces their affinity for leghemoglobin. Isobutyric acid binds only weakly to ferric leghemoglobin (K_{diss} ca. 500 mM at pH 5.3 and 20°C, as determined by the spectrophotometric method described in (16))and lactic acid appears not to bind at all. Thus it appears that whilst the heme pocket of ferric leghemoglobin is more open than that of ferric myoglobin, allowing the binding of long chain carboxylic acids, steric constraints within the pocket are still important and influence the nature of the metal-ligand bond.

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