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THE HAEM-ACCESSIBILITY IN LEGHAEMOGLOBIN OF *LUPINUS LUTEUS* AS OBSERVED BY PROTON MAGNETIC RELAXATION

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Using the solvent-protons' longitudinal magnetic relaxation rates (p.m.r.) for Lupinus luteus *leghaemoglobin derivatives the accessibility of the haem has been evaluated by our "stereo-chemical p.m.r. titration" method with nonexchangeable protons of aliphatic lower alcohols in otherwise deuterated solutions. The haem in leghaemoglobin is more accessible and its protein environment more flexible compared with vertebrate haemoglobins. The correlation time in aquometleghaemoglobin aqueous solution has been determined by measuring the frequency dispersion of the p.m.r. rates between 6.1 and 93 MHz. Taking into account the measured value of* $\tau_c = (7.7 \pm 0.5) \times 10^{-10}$ *s the iron-to-proton inter-spin distances have been calculated. The significance of these distances as well as the electronic g-factor anisotropy for elucidation of fine structural details of the haem-environment are discussed.*

The reversible oxygenation and low auto-oxidizability of the ferrous iron in the oxygen-carrying haemoproteins is regarded as being due to the hydrophobic pocket of the protein matrix surrounding the haem (1,2). Such a structural domain has been verified by X-ray crystal structure analysis for the vertebrate myoglobin (3) and haemoglobin (4) and in three phylogenetically different haemoproteins (5,6,7,8). A rather wide phylogenetic span has been encompassed in these studies, but all these haemoproteins transport oxygen in one way or the other, notwithstanding great differences in the type of their reversible oxygenation. The function of plant haemoglobins is involved in the nitrogen-fixation mechanism, most probably by facilitating the oxygen-diffusion (9). Recently, the three-dimensional structure of leghaemoglobin from root nodules of the yellow lupin, *Lupinus luteus* L. has been determined to a resolution of 5 Å (10). The general fold of the polypeptide chain turned out to be very similar to all the other oxygen-carrying haemoproteins so far analysed. The low resolution does not yet allow the haem-environment to be specified in more detail. Aggarwal & Riggs (11) consider it to be similar to those in other haemoproteins, on the grounds that the primary sequences of *soybean* leghaemoglobin (12) and the vertebrate ones have large homologies in the hydrophobic region expected for the haem-pocket. Appleby (13), on the other hand, advanced the hypothesis that because of much higher auto-oxidizability the haem in leghaemoglobin is not likely to be embedded deeply in a hydrophobic crevice. Such a view has been substantiated by Ellfok (14) who used various fatty acids and found that they all combined easily and firmly with soybean leghaemoglobin, implicating a rather accessible haem-iron as distinct from vertebrate myo-

globin which did not react with these ligands at all.

It therefore seemed appropriate to examine the accessibility of the haem in the *L. luteus* leghaemoglobin. The method used was, in essence, a "stereochemical titration", a term descriptive of the approach by Chance (15), Pauling (16) and Ellfolk (14), only this time applied to the proton magnetic relaxation (p.m.r.) technique. The difference, and its partial advantage, is that the p.m.r. method may yield, in addition to dynamic parameters, some direct structural data, like distances between the interacting species. These are, in a haemoprotein solution, the paramagnetic dipole moments of the iron-ion in the protein and the nuclear (proton) spins of the solvent. While the direct "stereochemical titration" is applicable only if the ligand can be bound, our method of using stereochemical markers with the relaxing protons (17) can be used whenever there is a protein crevice, so that either protons, or molecules of water, methanol, ethanediol or propanetriol, each approach the paramagnetic metal ion in a different way, but without bonding to it. The p.m.r. stereochemical titration with lower aliphatic alcohols was found to be an almost non-perturbing method for some haemoproteins, but the results with leghaemoglobin indicate that it is more susceptible to slight conformational changes under our experimental conditions. At any rate, the method does yield an overall view of a particular conformational domain in the vicinity of the paramagnetic centre. The presence of such a centre, on the other hand, severely restricts the applicability of the method.

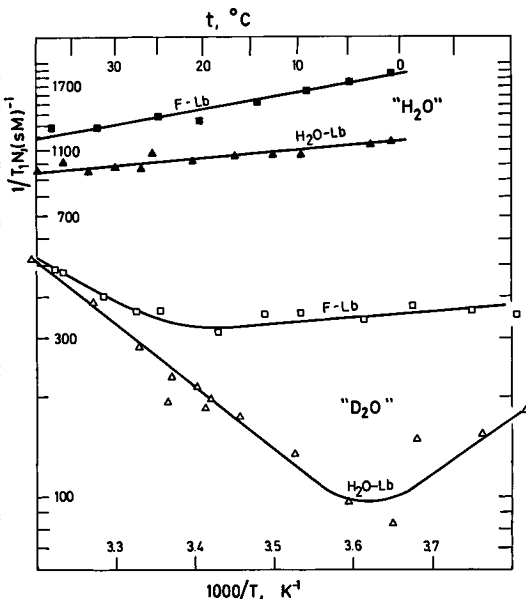

FIGURE 1

The temperature dependence of the longitudinal p.m.r. rates, at 24 MHz, in leghaemoglobin solutions. Filled symbols, ordinary aqueous solutions; open symbols, deuterated solutions containing 35% (v/v) $C_2H_4(OD)_2$. Squares, fluorometleghaemoglobin; triangles, aquometleghaemoglobin.

MATERIALS AND METHODS

The major fraction of leghaemoglobin from *L. luteus* was isolated as described previously (18). To compare the results with our earlier studies, the acetate buffer was replaced by dialysis against a solution of 0.05 M TRIS and 5×10^{-4} M EDTA, pH 6.4 containing either 0.1 M NaCl (filled triangles in Figs. 1 and 3), or 0.2 M NaF (filled squares in Fig. 1). For the measurements denoted by filled symbols in Fig. 2, propanetriol was added to the above solutions to give a final concentration of 20% (v/v). Deuterated solutions (open symbols in Figs. 1 and 2) were prepared by multiple dialysis against solvents containing either 0.1 M NaCl (triangles) or 0.2 M NaF (squares), measured pH 6.4 in D_2O. To deuterated solutions (Fig. 1) deuterated ethanediol was added with a final concentration of 35% (v/v), or 20% (v/v) deuterated propanetriol (Fig. 2). The details of the deuteration procedure are given in ref. 17. All the chemicals were reagent grade and used without further purification.

The protein concentrations were determined by the cyanomet-method (19) assuming $\varepsilon_{540}^{1\,cm} = 11 \times 10^3\ M^{-1}$. All the samples were centrifuged at 15,000 *g* for half an hour before p.m.r. measurements.

The temperature dependence of the solvent-protons longitudinal relaxation rates was measured at 24 MHz and the data evaluated as described previously (17). The frequency dispersion experiment was performed at 20±0.5°C between 6.1 and 93 MHz using a Bruker SXP pulsed spectrometer, as before (20).

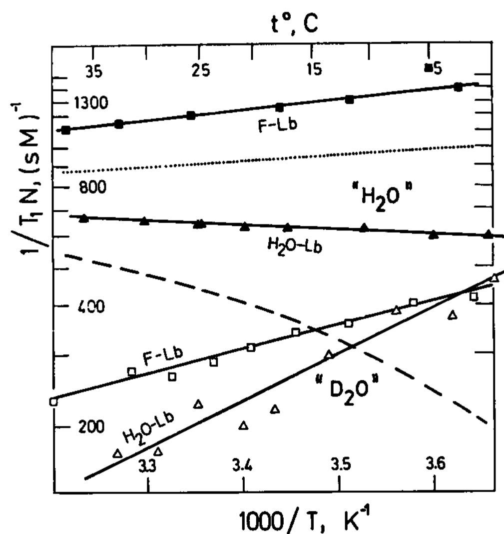

FIGURE 2

The temperature dependence of the longitudinal p.m.r. rates, at 24 MHz, in leghaemoglobin solutions. The ordinary aqueous solutions (filled symbols) contained 20% (v/v) $C_3H_5(OH)_3$, while the deuterated ones (open symbols) had 20% (v/v) $C_3H_5(OD)_3$. Squares, fluorometleghaemoglobin; triangles, aquomet derivative; dotted line, the pure paramagnetically induced contribution to the p.m.r. rates due to the presence of fluorometleghaemoglobin; dashed line, the same p.m.r. rate induced by aquometleghaemoglobin.

RESULTS AND DISCUSSION

There are three types of relaxation mechanisms which determine the temperature dependence of the proton magnetic relaxation rates in haemoprotein solutions (17). Each of them was observed in the present study and we shall deal with each in turn as we proceed with the discussion of Figs. 1 to 3.

In Figs. 1 and 2 the relaxation rates normalized per haem concentration are plotted in Arrhenius-type graphs. The squares and the triangles refer to the rates measured in fluorometleghaemoglobin (F-Lb) and in aquometleghaemoglobin (H_2O-Lb) solutions, respectively, after deduction of the rates measured in the corresponding solvents alone. The full symbols indicate the measurements in ordinary aqueous solutions, i.e. the ordinates refer to the relaxation rates ("H_2O") as observed through the proton magnetic relaxation of *all* the protons in the solvent: from the water molecules (Fig. 1) and from these molecules together with the methylene and hydroxyl protons of propanetriol (20% (v/v) added, Fig. 2). The open squares and triangles refer to measurements in completely deuterated solvents ("D_2O") with either 35% of ethanediol (Fig. 1) or 20% of propanetriol added (Fig. 2). In these "D_2O"-experiments only the relaxation of the aliphatic protons from the added marker molecule is measured. The relaxation rates in the former case (Fig. 2, "H_2O") had to be corrected for the

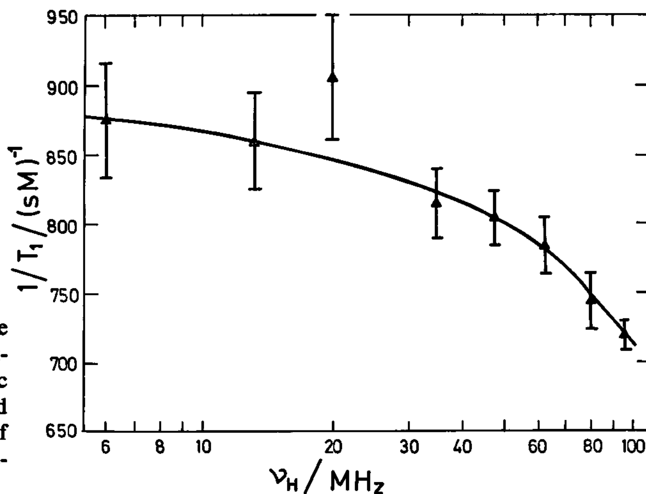

FIGURE 3

The frequency-dispersion of the paramagnetically induced solvent-proton longitudinal magnetic relaxation rates between 6.1 and 93 MHz in aqueous solution of aquometleghaemoglobin measured at 20±0.5°C.

"dilution" of chemically exchangeable protons owing to the presence of the aliphatic protons from propanetriol. These nonexchangeable protons "sense" the influence of the ferric haem-iron only via the "long-range' dipole-dipole interaction (see later) and contribute to the overall relaxation rate only slightly. This rate is given by

$$\frac{1}{NT_1} = \frac{n}{N_w} \cdot \frac{1}{T_{1M}} \qquad (1)$$

where $1/NT_1$ denotes the *molar* paramagnetic relaxation rate, n is here a constant (but see later), while N_w stands for the molarity of the species which relaxes with T_{1M} in the sphere of closest approach to the iron-ion. The value of N_w is lowered by the addition of any chemically nonexchangeable protons which do not enter the immediate neighbourhood of the iron-ion, and therefore the corrected $1/NT_1$ values in our case (Fig. 2, full symbols) are 12% larger than the originally measured relaxation rates.

As with vertebrate and other fluoromethaemoglobins (17), fluorometleghaemoglobin (F-Lb) in the present study shows the so-called fast-exchange relaxation within the whole of the available temperature region studied, in both the ordinary aqueous solutions (Fig. 1) and in those to which 20% (v/v) of ordinary propanetriol was added (Fig. 2). Such a temperature dependence is usually characterized by a positive slope in this graph and rather high relaxation rates. The two Arrhenius straight lines for the F-Lb in ordinary aqueous (Fig. 1) and in the aqueous solution with the propanetriol added (Fig. 2) are not identical, but they differ to a lesser extent than the corresponding pair of data for the H_2O-Lb.

The curves obtained with deuterated ("D_2O") F-Lb solutions, in Figs. 1 and 2, show considerably lower relaxation rates due to the only observable relaxation of the aliphatic protons from the added ethanediol or propanetriol. There is no doubt, therefore, that the dominant relaxation mechanism in ordinary aqueous F-Lb solutions ("H_2O") is indeed a very fast exchange of water molecules between the vicinity of the paramagnetic iron and the bulk of the solvent (17). These fast-exchange rates for F-Lb are shown by the dotted line in Fig. 2 as a result of subtracting the contribution of the outer-sphere relaxation (17) which is that measured in the "D_2O" experiment with propanetriol. The same procedure for the data of F-Lb in Fig. 1 is not strictly permissible because in the "H_2O" measurements no ethanediol was added, while the "D_2O" contained 35%, measuring thus its aliphatic-protons relaxation. It is also obvious from comparison of the "D_2O" curves for F-Lb in Figs. 1 and 2 that the outer-sphere relaxation is different. The inescapable conclusion is that, contrary to our experience with vertebrate haemoproteins, in the p.m.r. stereochemical titration for fluorometleghaemoglobin the addition of the alcohol markers induces a definite, though not dramatic, conformational change around the haem-iron. This domain of the protein structure appears, therefore, to be less rigid compared with the vertebrate haemoglobins.

The important difference between the F-Lb "D_2O" measurements with ethanediol and propanetriol (Figs. 1 and 2) is that in the case of propanetriol, throughout the whole of the available temperature range, the aliphatic protons of the marker molecule sense the paramagnetism of the haem-iron only via the "long-range" dipole-dipole interaction from the outer sphere. In other words, molecules of propanetriol do not approach the haem-iron closely enough to defined positions from which they would keep fluctuating with the bulk solvent. (They are, in fact, part of the bulk solvent itself.) Further, it means that the propanetriol influence of the rates in the "H_2O"-experiment has nothing to do with direct interaction between it and haem-iron (as confirmed by optical spectroscopy as well), but rather indirectly through a change in the dielectric constant of the solvent and/or different hydration sheath.

The "D_2O" data for F-Lb (Fig. 1) indicate the onset of an observable increase in the relaxation rates above some 20°C due to the thermally activated process of ethanediol exchange between the close vicinity of the haem-iron and bulk solvent. This is the first time that such an effect for a fluoromethaemoprotein has been observed. In the case of human A fluoromethaemoglobin, only the smallest marker molecule, that of methanol, exhibited even the fast-exchange relaxation mechanism, but ethanediol and propanetriol only showed the outer-sphere mechanism (17). Hence, judging by this dynamic aspect

of the p.m.r. stereochemical titration, the haem-iron in fluorometleghaemoglobin is more accessible than in vertebrate haemoglobins.

The most striking confirmation of the latter conclusion can be found in the difference between the "H_2O" and "D_2O" measurements for H_2O-Lb (Fig. 1) proving the very fast exchange of water molecules for the ordinary aqueous solution ("H_2O"). However, the "D_2O" curve, which reflects the relaxation rates only of the aliphatic ethanediol protons, means that above 5°C even the (larger) *molecules of the marker* exchange between the close vicinity of the haem-iron and bulk solvent fast enough to be observed by p.m.r. This, again, implies an accessible iron-ion in H_2O-Lb, an accessibility which is diminished in solutions containing propanetriol, as evidenced by the change from the fast to the temperature-limited exchange in Fig. 2 (dashed line) for the *water* molecules. On the other hand, as mentioned above, this change of mechanism implies a more flexible (or "plastic") protein environment of the haem in Lb compared to the vertebrate Hb's.

This qualitative analysis shows the following dynamic behaviour of the stereochemical markers:

(A) Fluorometleghaemoglobin

(i) water molecules are exchanged very quickly from the immediate vicinity of the haem-iron into bulk solvent.

(ii) above 20°C, ethanediol molecules fluctuate between the two environments in a temperature-activated manner.

(iii) propanetriol senses the haem-iron only outside the sphere of closest approach to it, without any indication of a temperature threshold as in the case of ethanediol.

(B) Aquometleghaemoglobin

(i) water molecules also fluctuate very quickly between the two environments, which is one of the few such instances observed so far (21,17).

(ii) in the presence of 20% propanetriol the exchange of water protons is slowed down, so that the relaxation is in the thermally activated range of exchange.

(iii) ethanediol enters the sphere of the haem-iron's immediate neighbourhood, its aliphatic protons showing the temperature-controlled exchange rate above 5°C.

(iv) propanetriol senses the haem-iron only from the outer-sphere.

The overall conclusion is that the haem-iron of both fluoro- and aquometleghaemoglobin is much more accessible to solvent molecules than any of the haemoproteins investigated thus far by this method (22). While a "stereochemical threshold" in the vertebrate haemoglobins lies between the molecule of methanol and ethanediol, here it is increased to a size between ethanediol and propanetriol.

The changes induced by the presence of propanetriol (and to a lesser extent, probably, by ethanediol) are smaller for the fluorometleghaemoglobin for which the type of the relaxation mechanism (very fast-exchange) does not alter, while it does (see above B/ii) in solutions of aquometleghaemoglobin. This may be an indication of a more stable structure when the fluoride ion is bound to the haem-iron which is almost completely in its high-spin state as opposed to the aquometform which is a mixture of high- and low-spin states (23).

The proton relaxation rates in haemoprotein solutions depend on the spatial disposition of the interacting spins of the haem-iron and solvent protons. When there is a fast exchange of these protons between a site in close vicinity of the paramagnetic iron-ion and bulk solvent, as proved here by the difference between "H_2O" and "D_2O" p.m.r.-data, the well-known Solomon equation (24) may be used to calculate the inter-spin distance, r (for details see ref. 17). The abbreviated form of the pertinent equation reads (with $\omega_{I,S}$ = nuclear (I), electron (S) Larmor frequencies):

$$(1/T_{1M}) = K_{I,S}\, n\, r^{-6}\, F(\omega_{I,S}, \tau_C) \qquad (2)$$

(I/T_{1M}), the p.m.r.-rate of the "bound" protons, can be obtained if, from the total solution relaxation rate, such as the "H_2O" data in Fig. 1 or 2, the outer-sphere relaxation ("D_2O" measurement) is subtracted with subsequent normalization by the metal-ion concentration. As mentioned before, this may be done in our case for Fig. 2, but not for Fig. 1. However, to the first approximation, due to rather low values of the outer-sphere relaxations and the expected smaller change if ethanediol was added in the "H_2O" experiments, we shall also subtract the outer-

sphere contribution (with 320 $s^{-1}M^{-1}$ for F-Lb and 100 $s^{-1}M^{-1}$ for H_2O-Lb) from Fig. 1 data. There are two more unknowns on the right-hand side of eq. 1 apart from the distance, r, which is sought for, while $K_{I,S}$ contains the known basic physical constants for the electron and nuclear spins. The number of the fluctuating nuclear spins (protons or the whole water molecules), n, will be dealt with later in this discussion. The last unknown, the correlation time, τ_C, determines the correlation function for the modulation of the dipole-dipole interaction of the two kinds of spins, with the known Larmor frequencies given by the instrumental set-up. Under the assumption that τ_C itself is frequency independent, the correlation function may be used to analyse the experimentally determined frequency dependence of the fast-exchange relaxation rates.

Such measurements were performed for the aquometleghaemoglobin in ordinary aqueous solution at 20°C and the results are reproduced in Fig. 3. The diamagnetic dispersion contribution was first deducted by using the data for carbonmonoxymyoglobin solution (20). The ordinates comprise the outer-sphere relaxation which is inherently measured together with the fast-exchange contribution under our experimental conditions. This does not really matter for the determination of τ_C, because the identical correlation function applies to both types of the relaxation mechanism but for possible differences in τ_C, see ref. 25. (The outer sphere is discussed later in relation to the determination of r.)

The dispersion curve drawn through the experimental points was obtained by an iterative computer fitting procedure of the Solomon equation (24) taking into account (25) Sternlicht's (26) consideration of the anisotropic g-tensor for the high-spin haemoproteins. In point of fact, and by analogy with the soya aquometleghaemoglobin (23), our H_2O-Lb is expected to have about 60% of the high-spin component at room temperature, at which the dispersion measurements were carried out. As the low-spin iron-ion contribution to the relaxation rates may be neglected, the dispersion curve in Fig. 3 is, in fact, due to the high-spin haem-iron in our sample of H_2O-Lb. The obtained $\tau_C = (7.7 \pm 0.5) \times 10^{-10}$ s is quite a reasonable value compared with the corresponding one for aquomet-Hb and Mb (20,27) of about 1.5×10^{-10} s. We have not measured the dispersion for the fluorometleghaemoglobin, but judging by the difference in τ_C between the two forms of human methaemoglobin (25,27), τ_C for F-Lb would be close to 1×10^{-9} s. As already discussed elsewhere (20,25) such a value of the correlation time means that it is actually the relaxation time of the electron spins of the high-spin haem-iron.

Using the above determined and estimated values of τ_C, and taking into account the high-spin content in the H_2O-Lb, while that of F-Lb is assumed to be 100% high-spin (23), the values of r are calculated by Solomon's formula (24) for different numbers of fluctuating protons assuming the direction of their fluctuation along the z-axis of the haem-plane, i.e. with $g = 2$, or within the x-y haem-plane, i.e. with $g = 6$. The data are summarized in Table 1.

TABLE 1

The iron-to-proton(s) distances, r (Å), derived from the p.m.r. data of Fig. 1, for the aquomet- and fluorometleghaemoglobin (H_2O–Lb and F–Lb) in the fast-exchange condition, at 20°C, with different number (n) *of fluctuating protons, and directions* (g = 2, *or* 6)

	H_2O–Lb $\tau_C = 7.7 \times 10^{-10}$ s		F–Lb $\tau_C \simeq 10^{-9}$ s	
	$(1/T_1)_{total}$			
	[a]1717 s^{-1} M^{-1}		1400 s^{-1} M^{-1}	
	$g = 2$	$g = 6$	$g = 2$	$g = 6$
$r_n = 1$	3.89	4.71	4.20	6.10
$r_n = 2$	4.37	5.28	4.71	6.80
	$(1/T_1)_{total} - (1/T_1)_{outer\ sphere}$			
	[a]1550 s^{-1} M^{-1}		1050 s^{-1} M^{-1}	
	$g = 2$	$g = 6$	$g = 2$	$g = 6$
$r_n = 1$	3.96	4.79	4.40	6.35
$r_n = 2$	4.44	5.37	4.94	7.13

[a] These relaxation rates are normalized to 100% high-spin.

A general survey of the data in Table 1 shows that, once the fast-exchange relaxation mechanism had been proven by the difference between the "H_2O" and "D_2O" measurements, the deduction of the outer-sphere contribution is not of great importance in view of the r^{-6}-dependence in eq. 2. Besides, as Sternlicht (25)

points out, even the $K_{I,S}$ – though comprising only the basic physical constants – is not completely devoid of uncertainty because of *g*-tensor anisotropy, which may amount to a factor of two. The following discussion will show that the calculated values of *r* in Table 1 are very useful in considering the general structural characteristics of the haem-environment, but cannot define its strict structural details.

In spite of a "simple" and rather well-defined haem-iron neighbourhood in haemoproteins, the number of the fast-exchanging protons (*n* in eq. 2 and Table 1) is still under discussion. As it is of importance to evaluate first the *smallest* possible distance from the iron-ion of one exchanging proton, we shall consider the values of *r* for that case ($n = 1$) in Table 1 obtained from the *total* relaxation rates.

The position of the sixth-ligand water molecule has been verified by crystal structure analysis for vertebrate haemoproteins (3,4) and *Chironomus* haemoglobin (5,6), but not yet for the aquometleghaemoglobin. There is, however, every chance that it may be at the same position here, too, which means that its protons are at most 3.0 Å (2.6 Å in myoglobin) apart from the iron-ion. Data from Table 1 definitely exclude the possibility that the protons of this water molecule are responsible for the fast-exchange relaxation observed with H_2O-Lb, because the shortest *r* determined is $\geqq 4$ Å. This is in accordance with earlier findings of ours (17) supporting the hypothesis (28) that other (water) protons which came close to the iron-ion fluctuate between the two environments. A reconsideration of the results for fluoromethaemoproteins (25) together with the pertinent data in Table 1 in the light of Sternlicht's formula suggests that the same is true for such liganded haemoproteins. In other words, the fluctuating proton is not (22) hydrogen bonded to the fluoride ion (29). A further analysis based on the known structural details within the haem-pocket in vertebrate and in *Chironomus* haemoglobin (22) excludes the possibility that the fast-exchange rate can be accounted for by one single proton fluctuating from any possible dissociable site of an amino acid residue. The conclusion was reached that water molecules carry the fluctuating protons. Now, in the case of leghaemoglobin, it is not possible to draw a firm conclusion, but the whole behaviour of this haemoprotein in the present study suggests very flexible and open haem surroundings, which is in complete agreement with Ellfolk's finding (14). There is, therefore, every chance that more than one water molecule ($n = 4$, or even 6) could be accommodated around the haem-iron to *specific* positions from which they fluctuate with bulk solvent. If this were true, an average *r* is estimated to be about 6 to 7 Å. In vertebrate and insect haemoglobins this amounts to the "mouthpiece" of the haem-pocket from two possible directions of approach towards the iron-ion. That this distance is still within the protein matrix surrounding the haem in leghaemoglobin and not within the space of free solvent is shown by the difference in "H_2O" and "D_2O" measurements with both ethanediol (a) and propantriol (b) as markers. In the first case (a) there would not have been any difference in the measured relaxation rates were the exchanging protons in fact at the border line of the outer sphere. In the case of propanetriol (b), the induced change of the relaxation mechanism equivalent to a slowing down of the proton exchange could only result if the positions of the exchanging protons in the ordinary aqueous solution (the "native" state) are at sites defined by the haem-pocket protein-matrix.

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