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THE MOLECULAR MECHANISM OF THE TEMPERATURE ENHANCEMENT OF PROTON MAGNETIC RELAXATION RATES IN METHAEMOPROTEIN SOLUTIONS

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The mechanism of water exchange between the haem-pocket and bulk solvent in aqueous methaemoprotein solutions was firmly substantiated by using the aliphatic protons of certain lower alcohols in an otherwise deuterated solution for measuring the incremental relaxation rates resulting from their magnetic interaction with the haem-iron. The fast-exchange condition was established for solutions of horse fluorometmyoglobin, human A fluoromethaemoglobin and Chironomus thummi aquomethaemoglobin. The distances between the exchangeable protons and the haem-iron obtained from these PMR measurements concur with the presence of the fluoride ion, while for Chironomus aquomethaemoglobin this distance is also much larger than that resulting from the location of the 6th site water molecule. The latter finding is the first clear-cut evidence that the exchanging protons belong to the next neighbour water molecule, a previously advanced hypothesis. The exchanging water molecule may thus serve as a natural probe for comparing the haem-pocket conformational state(s) under different conditions or in various haemoproteins.

1. Introduction

Met(ferri)haemoproteins contain an intrinsic paramagnetic probe, the ferric iron of the haem group. It is secluded within a part of the protein matrix, the haem-pocket, which is structurally well defined [1,2]. One would therefore hope that the finer details of the conformational state(s) of the haem-pocket under different conditions could be delineated by the proton magnetic relaxation (PMR) technique. In view of this the prime value of this method is in the structural parameters which in principle it may yield. The first is the distance of closest possible approach of solvent protons towards the haem-iron outside its immediate neighbourhood. Because of the shape of the haem-pocket, the relevant "outer-sphere" relaxation rate can only be used as an overall parameter of this accessibility, but no particular distance could be specified [3]. The second structural parameter is the distance, r, from the paramagnetic haem-iron to the protons which occupy a fixed site nearby but interchange with the bulk solvent at a rate, $1/\tau_B$, much larger than the longitudinal magnetic relaxation rate of the exchanging protons, $1/T_{1B}$. The values of r derived in our previous work [3,4] for the aquomethaemoproteins may thus be subject to criticism because the above condition was not satisfied.

Derzhansky et al. [5] interpreted their temperature study of PMR rates in a solution of dolphin metmyoglobin in a completely different way. Atanasov [6] used this alternative interpretation as, indeed, the only evidence of a structural kind in developing the hypothesis of "predenaturational conformation changes" as a functional mechanism for haemoproteins. In contrast to the "proton-exchange" idea which tacitly assumes no changes of protein conformation with temperature, the other hypothesis states that the water molecules with their protons are immobilized within the haem-pocket, but a shift of the haem towards the protein surface is assumed.

The purpose of this paper is twofold:

(i) to show the physical reality of the proton-exchange mechanism in the proton magnetic relaxation of methaemoprotein solutions, and,

(ii) to describe those cases in which the fast-exchange condition can be proven beyond doubt so that reliable iron–proton distances can be derived.

Our approach was to make use of inert marker molecules, larger than the molecule of water, bearing

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chemically non-exchangeable aliphatic protons. In a fully deuterated solution of methaemoproteins the aliphatic protons of a sufficiently large marker molecule will sense the paramagnetic iron only via the "outer-sphere" relaxation, because they cannot enter the haem-pocket. If they do, the rate of their switching between the pocket and bulk solvent would be low enough not to affect the overall relaxation. Hence, the test of the "conformation" [6] hypothesis is the comparison of the relaxation rates in a solution fully deuterated, except for the aliphatic protons, with those rates measured in a similar solution with a sufficient amount of hydroxyl or hydronium ions. The two sets of measurements should be practically identical if the "conformation" hypothesis is valid. If they are not identical the proton-exchange mechanism plays an important role, depending on the temperature and other conditions.

2. Materials and method

2.1. Haemoproteins and solvents

Human haemoglobin was prepared from fresh blood (from the local blood bank) following the method of Cameron and George [7]. The met(ferri)-form was obtained by the addition of potassium ferricyanide with subsequent extensive dialysis against deionized water. After the initial liophylization the methaemoglobin solution was liophylized three times from D_2O allowing more than 24 h for the H → D exchange between each liophylization. The liophylized and deuterated methaemoglobin was then dissolved in the relevant solvent (vide infra).

Horse metmyoglobin solutions were prepared by dissolving liophylized metmyoglobin (Calbiochem, lot No. 100660) in the relevant solvent. To make deuterated solutions, metmyoglobin was subjected to the same deuteration procedure as were the methaemoglobin samples.

Methanol, 1,2-ethanediol, and 1,2,3-propanetriol (all Merck, reagent grade) were distilled five times with D_2O (Fluka, 99.7%, or Prochem B.O.C., 99.8%). High resolution proton magnetic resonance spectra indicated less than 0.1% HDO relative to the peak of the non-exchangeable protons.

The experiments shown in fig. 1 were performed

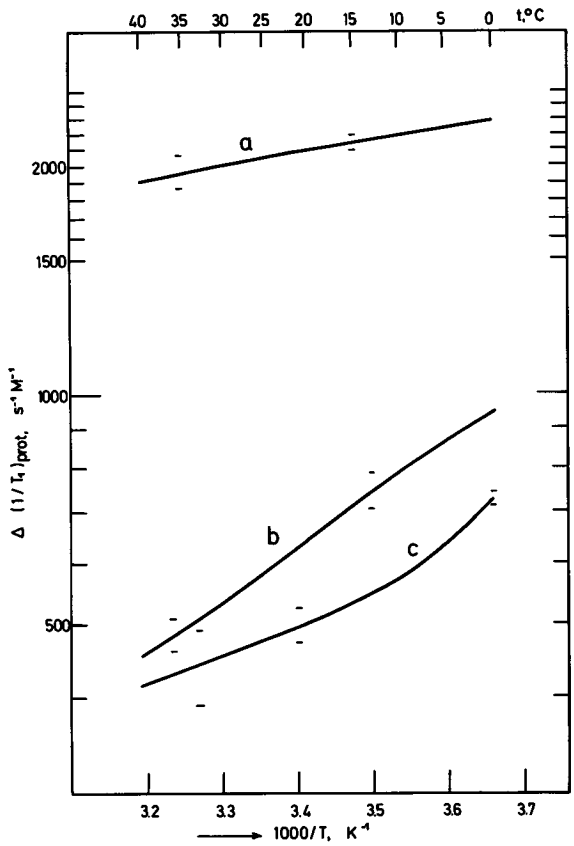

Fig. 1. The temperature dependence of the longitudinal magnetic relaxation rates of the solvent protons as increments induced per haem concentration of human A fluoromethaemoglobin dissolved in heavy water with addition of 2.95 M of: (a) CH_3^*OD, (b) $C_3H_5^*(OD)_3$, and (c) $C_2H_4^*(OD)_2$, where the asterisk denotes the solvent protons.

with equimolar (2.95 M) solutions of deuterated methanol, CH_3OD (curve a), 1,2-ethanediol, $C_2H_4(OD)_2$ (curve c), and 1,2,3-propanetriol, $C_3H_5(OD)_3$ (curve b). The protein concentrations were 5.05–5.27 mM (per haem). As in all the other experiments with fluoromethaemoproteins, the solutions contained 0.2 M sodium fluoride (Merck) in H_2O or D_2O. The measured pH was between 5.8 and 6.2 for reasons explained before [3].

Fluoromethaemoglobin and fluorometmyoglobin solutions (figs. 2 and 3, respectively) were prepared by dissolving the methaemoproteins in 0.2 M NaF + 5×10^{-4} M EDTA + 37.2 w% $C_2H_4(OH)_2$ in H_2O, pH 5.8, and in the case of the deuterated solution in 0.2 M NaF + 35.0 w% $C_2H_4(OD)_2$ in D_2O, measured

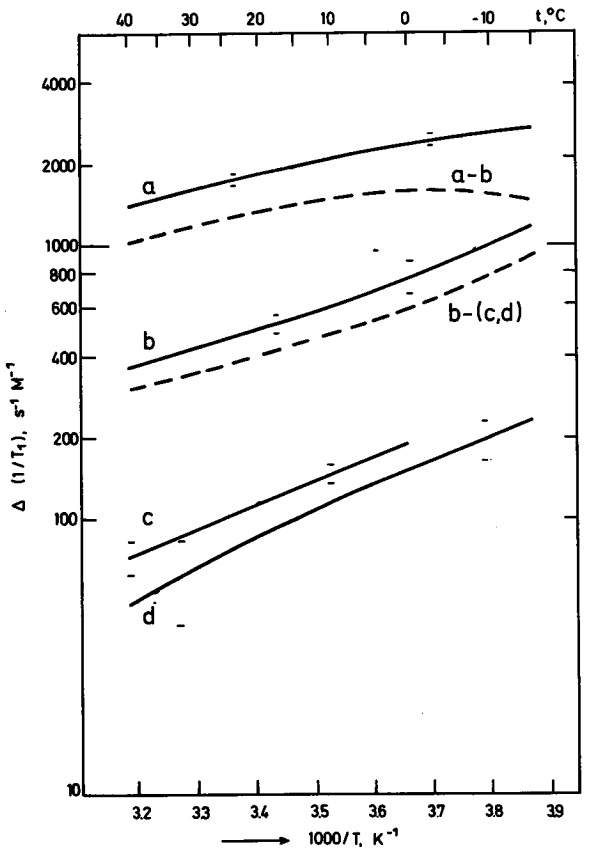

Fig. 2. The temperature dependence of the PMR rates as in fig. 1 induced by human A fluoromethaemoglobin and carbonmonoxyhaemoglobin in mixtures of water and ethanediol. (a) FluorometHb in 37.2 w% $C_2H_4^*(OH^*)_2 + H_2^*O$, and (b) $C_2H_4^*(OD)_2 + D_2O$. (c) COHb in 35.0 w% $C_2H_4^*(OD)_2 + D_2O$ and (d) in 37.2 w% $C_2H_4^*(OH^*)_2 + H_2^*O$.

pH 6.4. The carbonmonoxyhaemoglobin samples (fig. 2) were prepared by reduction of methaemoglobin with sodium dithionite under a CO stream with subsequent dialysis against either 0.1 M NaCl with 35.0 w% $C_2H_4(OD)_2$ in D_2O (curve c) or 0.1 M NaCl with 37.2 w% $C_2H_4(OH)_2$ in H_2O (curve d), the latter being reproduced from ref. [8]. Curve (c) in fig. 3 was obtained with a solution of horse CO myoglobin in 0.1 M NaCl + 0.05 M Tris + 5×10^{-4} M EDTA + 37.2 w% $C_2H_4(OH)_2$, pH 5.8.

The methaemoglobin crystals from Chironomus thummi larvae originated from Dr. Huber's laboratory (Max Planck Institute, Munich). These crystals were dissolved and dialysed against 0.1 M NaCl + 5×10^{-4} M EDTA + 37.2 w% $C_2H_4(OH)_2$ in H_2O, pH 6.20, and 0.1 M NaCl + 35.0 w% $C_2H_4(OD)_2$ in D_2O, pH 6.25, respectively (fig. 4).

The human methaemoglobin samples in fig. 5 were obtained by dialysing the original, non-liophylized, methaemoglobin solutions against acid, pH 6.00 (curve Hb_{H_2O}), or alkaline, pH 9.65 (curve Hb_{OH^-}), 0.1 M NaCl + 0.05 M Tris + 5×10^{-4} EDTA solutions. The commercial, liophylized metmyoglobin was dissolved in the relevant solvent and dialyzed against it (curves Mb_{H_2O} and Mb_{OH^-}). Only the results represented by curve Hb_{OD^-} are due to a solution of the deuterated liophylized methaemoglobin in 0.1 M NaCl with 35.0 w% $C_2H_4(OD)_2$ in D_2O, pH_{meas} 9.40.

All the solutions were centrifuged at 15000 G for half an hour before PMR measurements.

The concentrations were determined using the cyanmet-method [9]. The examined haemoprotein solutions had spectra with the satisfactory ratios of maxima, which were also frequently checked after the relaxation measurements. The spectra in the visible range were taken for the described solutions with methanol, ethanediol, and propanetriol, and the maxima ratios of these spectra correspond to those published elsewhere [7]. The relaxation rates also show that the native state of haemoproteins was preserved under the described conditions (see section 4).

2.2. T_1-measurements

The proton magnetic longitudinal relaxation times, T_1, were measured by the π-t-$\pi/2$ pulse sequence. Variable t-delays were applied using a digital readout pulsed spectrometer (Jožef Stefan Institute, Ljubljana) with a high resolution magnet (Bruker-Physik, Karlsruhe), at 24 MHz, and also at 12 MHz for fluorometmyoglobin in H_2O. A computer programme yielded T_1 values, usually with a correlation coefficient not less than 0.99 for the least square straight lines with maximal errors in T_1 of 8%. The spectrometer was provided with a temperature controller and the temperature of the samples was kept constant within 1°C by a thermostated nitrogen stream. The actual temperatures of the solutions were checked by direct measurements with a calibrated thermistor probe (Yellow Springs, Ohio) before and after T_1 measurements.

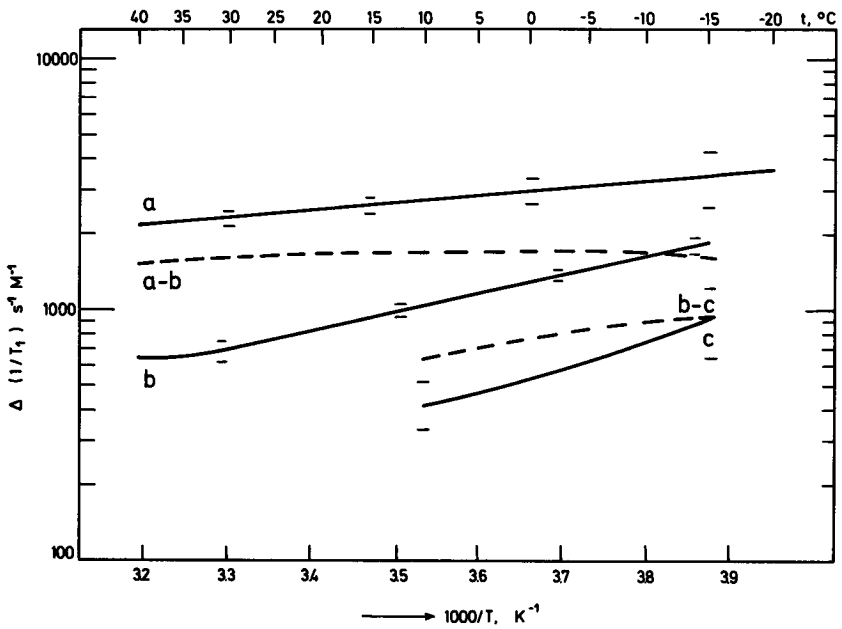

Fig. 3. The temperature dependence of the PMR rates for horse fluorometmyoglobin solutions as in fig. 2a, b, and c.

3. Results

3.1. Presentation of data

All the data presented in figs. 1 to 5 are plotted as the logarithms of the PMR rates versus reciprocal absolute temperature, with the linear temperature scale indicated as well. The relaxation rates, $\Delta(1/T_1)_{prot}$, are those induced by the magnetic interaction of solvent protons (those of H_2O *and* ethanediol, or only those from the aliphatic protons in "D_2O"-solutions) with the dissolved haemoprotein. They were obtained in the following way: the computer programme yielded the relaxation rates $(1/T_1)$ for the haemoprotein solution and its corresponding solvent. The relatively small concentration of protein allows to deduct the relaxation rates for the solvent directly from those of the haemoprotein solutions. The differences obtained, normalized per haem concentration, are plotted in the figures described below. For more accurate calculations of stereochemical parameters (see section 4) the correction was made for the finite volume of the protein in solution. The curves shown in this paper are smoothed, with the indicated error (unless it equals the line-width) obtained from the points of largest deviation in drawing the mean curves for the haemoprotein solutions and the solvents relaxation rates. The original T_1-data may be obtained on request.

3.2. Description of the figures

Fig. 1 represents the relaxation rate measured for the aliphatic protons, i.e., CH_3-protons of methanol, CH_2-protons of 1,2-ethanediol, and CH_2- and CH-protons of 1,2,3-propanetriol. The chemically exchangeable protons (from hydroxyls or hydronium ions) were eliminated by the deuteration procedures as described in section 2.1. The results suggest that the relaxation rates observed for the methyl protons of methanol are comparable with those obtained with ordinary aqueous solutions, while the rates measured for the other two marker molecules are much lower, and the smaller of the latter two markers, 1,2-ethanediol, was used in the next experiments.

This evidence was taken to prove that the relaxation rates of the CH_2-protons of ethanediol, measured in completely deuterated solutions, represent the so called "outer-sphere" relaxation. Figs. 2, 3, and 4 were therefore obtained from measurements with human fluoromethaemoglobin, horse fluorometmyoglobin, and Chironomus thummi aquomethaemoglobin,

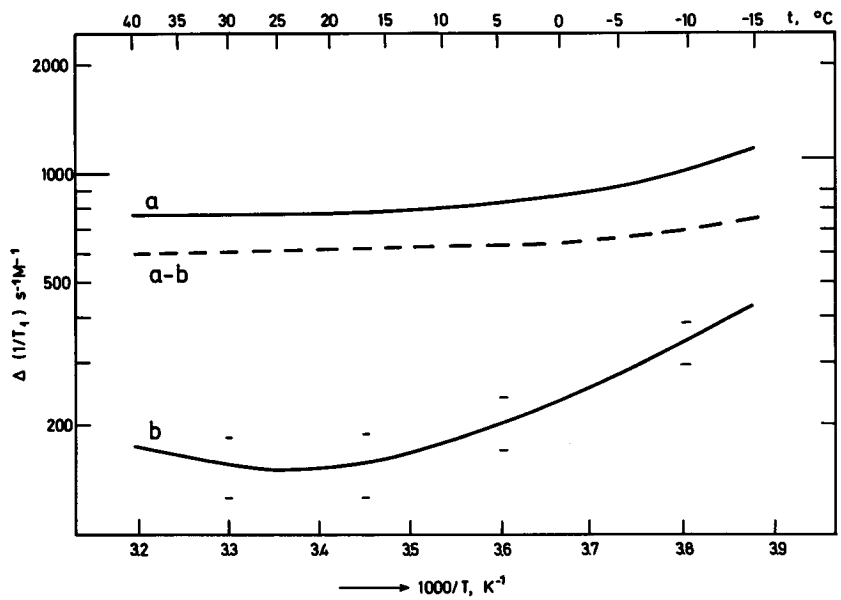

Fig. 4. The temperature dependence of the PMR rates for Chironomus thummi aquomethaemoglobin solutions as in fig. 2a and b.

respectively, in ordinary aqueous solutions with ordinary ethanediol added, and in deuterated solutions with only marker CH_2-protons present. The curves (a) in these figures refer to the first type of solutions, "H_2O", indicating the "fast-exchange" controlled relaxation rates, while those marked by (b) refer to the second type of solutions, "D_2O", induced by the "outer-sphere" relaxation. The difference in the relaxation rates between the two mechanisms is indicated in these figures by the broken lines marked (a – b), representing the pure, paramagnetic, "fast-exchange" relaxation rate. The "outer-sphere" relaxation, (b), is composed, again, of the pure paramagnetic part due to the dipole–dipole interaction of electron (haem-iron) and nuclear (proton) spins, and that due to the diamagnetic interaction between the solvent and protein protons. The latter contribution was measured for the carbonmonoxyhaemoglobin solutions using both "H_2O" and "D_2O" solvents. The results are represented by curves (c) and (d) in fig. 2 and curve (c) in fig. 3 for carbonmonoxymyoglobin in a "D_2O"-solution. The two sets of data almost coincide. Therefore, within the experimental error, the two solvents ("H_2O" versus "D_2O") do not significantly differ in their influence on the diamagnetic PMR rates. The difference between the curves (b) in figs. 2 and 3 and the mean diamagnetic relaxation rates equals the pure paramagnetic "outer-sphere" relaxation as represented in fig. 2 by the curve b – (c,d), or, for myoglobin, in fig. 3 (b – c).

Fig. 5 shows the comparison between the temperature dependence of methaemoglobin and metmyoglobin relaxation rates measured at different pH of the solvents (see section 2.1). In the first case both haemoproteins were in their aquomet-form, and in the second, in their hydroxomet-form. The latter form of methaemoglobin was also used in the "D_2O" measurements of hydroxomethaemoglobin (curve Hb_{OD}-) in order to compare them with those given by the curve Hb_{OH}-.

4. Discussion

4.1. Proton exchange

Methanol, ethanediol and propanetriol were used as the marker molecules in methaemoglobin aqueous solutions because they are practically inert towards this protein molecule as shown [10] by magnetochemical titration, and in other studies [8, 11–14]. Fluoromethaemoglobin was chosen for the comparison of

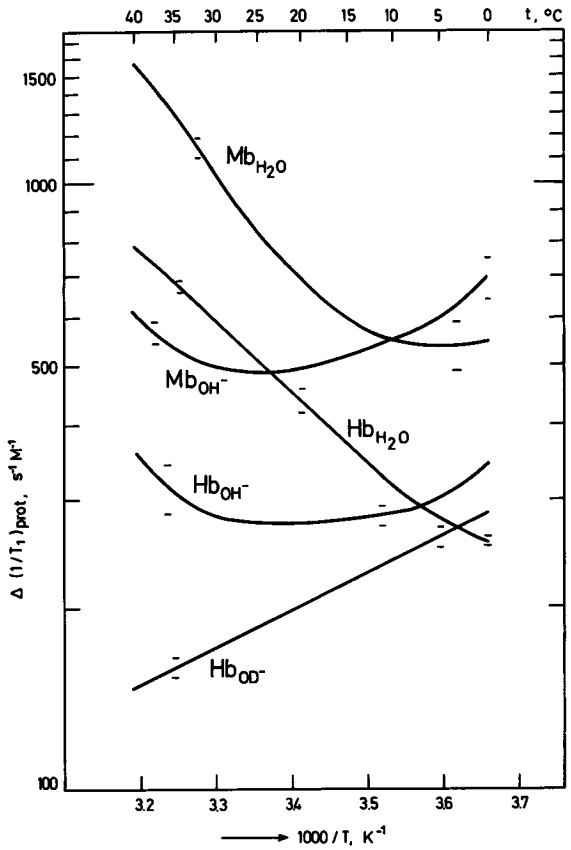

Fig. 5. The temperature dependence of the solvent protons longitudinal magnetic relaxation rates as increments induced per haem concentration in aqueous solutions of horse aquometmyoglobin, Mb_{H_2O}, and hydroxometmyoglobin, Mb_{OH^-}; human A aquomethaemoglobin, Hb_{H_2O}, and hydroxomethaemoglobin, Hb_{OH^-}. The Hb_{OD^-} curve was obtained with a solution of metHb in D_2O with 35 w% $C_2H_4(OD)_2$, pH_{meas} 9.40.

the three marker molecules because, like fluorometmyoglobin [15], its relaxation in aqueous solution seemed to be dominated by the mechanism of fast proton exchange between the haem-pocket and bulk solvent. On the other hand, if there is any slight interaction of aquomethaemoglobin with methanol [10], the haem-iron in fluoromethaemoglobin would be protected by the fluoride ion at the 6th site.

As no relaxation other than that of the aliphatic protons was measured in the experiments from fig. 1, one may conclude that there is a size limit between the molecules of methanol and ethanediol. While the methanol molecule can exchange between the haem-pocket and (deuterated) bulk solvent, ethanediol and propanetriol cannot. The small difference between the latter two may originate from differences in hydration sheaths around haemoglobin molecules in the two solutions, a point which requires further study. It is obvious that the "conformation" hypothesis is incompatible with these results, while they can be explained by the exchange mechanism. In the first case the (b) and (c) curves should in fact closely resemble that for methanol, (a). Because they do not, it must be concluded that they indeed represent only the "outer-sphere" relaxation, whereas the methanol curve also results from the much larger contribution due to the exchange mechanism.

We have also obtained results showing the transition from the purely "outer-sphere" relaxation [similar to (b) in fig. 1] through the partially to the fully-measured exchange contribution by varying the amount of exchanging protons in solutions of haemoglobin.

From such changes in the temperature dependence of the relaxation rates, the following conclusions can be drawn:

(i) There is indeed an exchange of protons between the haem-pocket and bulk solvent in solutions of aquomethaemoglobin. This does not imply that the fast-exchange condition (see Introduction) is fulfilled in this particular case, but the exchange is fast enough to dominate the overall relaxation rate at higher temperatures.

(ii) There is no shift in the conformational equilibrium for haemoglobin molecules on raising the temperature, of the kind postulated in references [5, 6]. Were there any such effect, curves like (b) in fig. 1 should have a negative instead of the observed positive slope with aquomethaemoglobin solution.

4.2. The iron–proton distance and the fast-exchange condition

Our experiments strongly support the physical reality of protons fluctuating between the haem-pocket and bulk solvent. It remains now to examine whether this proton exchange is fast enough for the existing theory to be applicable in calculating the distance, r, between the haem-iron and the nearest protons exchanging with bulk solvent. The only direct approach to this problem is to measure the residance

time, τ_B, of the protons in question inside the haem-pocket. This could be done by measuring the transverse relaxation time as a function of the pulse separation in a Carr–Purcell sequence, as applied for instance in the case of inorganic aquo-ions [16]. There are other, though less direct, criteria, by which one can define the fast-exchange conditions, and we shall apply them firstly to the fluoromethaemoproteins, and secondly to the aquomethaemoglobin from the Chironomus thummi larvae.

4.2.1. Fluoromethaemoproteins

We compare first our results for fluorometmyoglobin, fig. 3, with the similar measurements of Mildvan et al. [15]. As expected, the two sets of data are practically identical, and in agreement, again, with our experience that addition of ethanediol does not change the relaxation to any significant extent [8]. It should be pointed out that the curve (a) in fig. 3 represents the proton relaxation of a solution in which the protons can easily switch between the water and ethanediol hydroxyls and also between the haem-pocket and bulk solvent. Within the indicated error there was no difference between pairs of measurements at 24 and 12 MHz (the Larmor frequency used in ref. [15] was 24.3 MHz). It was hoped that by extending the measurements below zero centigrades, i.e., below the temperature limit in ref. [15], the curve (a) in fig. 3 would bend downwards owing to the diminished rate of proton exchange between the haem-pocket and bulk solvent. This would then seem to suggest, though not prove definitely, that the fast exchange condition, $T_{1B}/\tau_B > 10$, is maintained at the higher temperatures, as assumed by Mildvan et al. [15]. The expected curvature could not be detected even as low as –20°C. We therefore used our "marker method". In full confirmation of our data from fig. 1 for fluoromethaemoglobin, the "H_2O" (a) and the "D_2O" (b) curves in fig. 3 are quite different. This is strong experimental evidence for the "fast-exchange" at least above 0°C, because there is simply no other alternative (see the schematic fig. 6 in ref. [3]). Hence, the difference between the "H_2O" and "D_2O" curves, (a – b), represents the contribution to the relaxation from T_{1B}, the relaxation time of the exchanging protons while located near the iron inside the haem-pocket.

The values of $\Delta(1/T_1)_{a-b}$ from fig. 3 may be used to derive r, by the Solomon–Bloembergen equation [17], which, abbreviated, reads:

$$(1/T_1)_{a-b} = (n_h/N_p)(1/T_{1B}) = Kn_h F(\tau_C) r^{-6}. \quad (1)$$

$F(\tau_C)$ is a function of the correlation time, τ_C, for the dipole–dipole interaction of the haem-iron spin (= 5/2) and the proton spin (= 1/2). The most exact way of calculating r would be to first of all obtain τ_C for fluorometmyoglobin by measuring the frequency dispersion of $\Delta(1/T_1)_{a-b}$. Since we lack this data at present we take the value of $\tau_C = \tau_S$ (the electron spin relaxation time) = 1.5×10^{-10} s obtained in a study [18] of aquometmyoglobin at 5°C. While the fluoride complex is 100% high spin the aquomet may contain at 5°C a few percent of the low-spin form [19]. Because of this, and because of the different sixth ligands, (F versus H_2O) τ_S (= τ_C) in aquomet- and fluorometmyoglobin may not necessarily be identical. However, we assume that the two τ_C's are similar.

It is not definitely known how many protons [n_h in eq. (1)] from the haem-pocket of fluoromethaemoproteins exchange with bulk solvent. Mildvan et al. [15] seem to have assumed only one such proton. In agreement with these authors, who found r = 2.9 Å, we obtain $r_{1,MbF}$ = 2.99 Å. The difference of about 0.1 Å is due to our correction by the data of curve (b) in fig. 3. Using the same evaluation of the data for fluoromethaemoglobin (fig. 2), $r_{1,HbF}$ = 3.05 Å.

The question as to whether intact water molecules exchange between the two environments has been mentioned by Mildvan et al. [15] with regard to their measurements of ^{17}O-relaxation. However, no conclusion is attainable at all because ^{17}O-relaxation is not sensitive enough to the presence of paramagnetic ions. The pertinent factor is obtained from the magnetogyric ratios: $\gamma_{^{17}O}/\gamma_{^{1}H} = 0.018$, so that in order to obtain equal paramagnetic enhancement of ^{17}O-relaxation rates one would require concentrations of protein about 50 times the concentrations used in ^{1}H measurements. This is impossible to achieve.

If two protons of a water molecule were exchanging, r_2 will have a unique value only if the two of them were equidistant from the haem-iron. Thus, $r_{2,MbF}$ = 3.35 Å, and $r_{2,HbF}$ = 3.43 Å.

The asymmetric, Fe–F^- ··· HOH-bonded water molecule seems to be the most probable case (by extrapolation from pure ionic solutions, see ref. [20]). The observed relaxation rates for fluoromethaemo-

proteins then yield:

$r_{2,MbF,as}$: 3.03 Å and 4.55 Å;

$r_{2,HbF,as}$: 3.10 Å and 4.62 Å.

The latter values appear quite reasonable regarding the available space within the haem-pocket. This is also corroborated by deriving the iron-to-proton(s) distance(s) from the "methanol" results (see fig. 1, curve a – curve b), where $n_h = 3$. With the CH_3-free-rotation, an r_3 almost equal for all the three protons may be assumed, which results in

$r_{3,Hb} = 5.54$ Å.

It seems more likely that the methanol molecule faces the haem-iron with the CH_3-group, rather than with its OD-tail. For an asymmetric orientation the proximal proton of the CH_3-group will be at 5.05 Å, with the most distal proton at 6.29 Å from the haem-iron. Thus, with an approximate r of 5.5 ± 0.5 Å the CH_3-group of the methanol molecule is just about inside the haem-pocket but very close to its mouth-piece.

4.2.2. Aquomethaemoproteins

It may be concluded from fig. 4 along the same line of reasoning as for fluoromethaemoproteins that the proton magnetic relaxation of Chironomus aquomethaemoglobin solution is also governed by the fast exchange mechanism from +40°C down to at least −10°C. Further, using eq. (1) and the same numerical data as before, with $\Delta(1/T_1)_{a-b} = 620\ s^{-1}\ M^{-1}$ at 5°C,

$r_{1,Ch} = 3.53$ Å, $r_{2,Ch} = 3.96$ Å, or

$r_{2,Ch,as}$: 3.60 Å and 5.12 Å.

All these values are appreciably larger than the value 2.8 Å which follows from the fact that a molecule of water has been identified at the sixth ligand site of the haem-iron in the crystal structure analysis of Chironomus thummi aquomethaemoglobin [21]. The crystal structure data and our proton magnetic relaxation results obtained with the solution of this haemoglobin can be reconciled by the hypothesis [3,4] postulating the existence of protons exchanging with bulk solvent from a site inside the haem-pocket. This site is next to that occupied by the water molecule in the first coordination sphere of the haem-iron. The results from fig. 4 for Chironomus haemoglobin are in fact the first clear-cut evidence supporting this hypothesis. It is likely that the lamprey haemoglobin (Petromyzon marinus) fraction 2, as discussed in ref. [22], could be another such example.

We have not yet found a tetrameric aquomethaemoglobin with well defined "fast-exchange" relaxation mechanism under our experimental conditions. Fig. 5 illustrates the shape of the curves for solutions of mammalian methaemoproteins obtained under conditions comparable to other data in this paper. Pairs of measurements for horse myoglobin, a single unit protein like Chironomus, and for human haemoglobin A, a tetramer, both at pH close to either 6 or 10 are given. Neither the aquometmyoglobin nor the aquomethaemoglobin curves (pH 6) show any levelling off at the higher temperatures, which would be expected if the "fast-exchange" mechanism were to take over. In previous work carried out in this laboratory [3,4] it was assumed that the temperature and the corresponding relaxation rate of such a turning point is close to the highest available temperature in these measurements, i.e., 40°C. The result obtained with Chironomus haemoglobin is reassuring in that the basic conclusion about the exchangeable protons, which are not those from the water molecule in the first coordination sphere of the haem-iron, appears to be quite feasible in spite of the remaining doubts about the actual r-values for the aquomethaemoproteins of mammals.

The fluoromethaemoproteins are an example of the stereochemistry around the haem-iron with the sixth site occupied by a ligand without protons, the fluoride ion, and the exchangeable protons are from a next-neighbour site within the haem-pocket. Another such example was expected to be found in alcaline (pH ≈ 10) methaemoproteins in which the sixth ligand has long been assumed to be the hydroxyl ion hydrogen bonded to the imidazole nitrogen of the distal histidine. However, the results from fig. 5 (compare the Hb_{OH}- and Hb_{OD}- curves) clearly show that within the available temperature range the exchange of protons in hydroxomethaemoprotein is far from having the "fast-exchange" relaxation rate. For aquomethaemoglobin and aquometmyoglobin the contribution of the exchange mechanism becomes observable beyond the "outer-sphere" relaxation above

0°C and above 10°C, respectively. This temperature is raised to some 30°C for the hydroxometforms of both these proteins. The solutions of the latter exhibit therefore mostly the "outer-sphere" relaxation mechanism. Hence, comparisons between the relaxation behaviour of the two met-forms with the aim of drawing stereochemical conclusions as has been done in ref. [15] do not seem justified. The near coincidence at lower temperatures of the "H_2O" and "D_2O" curves for the hydroxomethaemoglobin solutions is yet additional evidence that under the conditions of our marker method the relaxation rates measured via the methyl protons of ethanediol do properly represent the "outer-sphere" relaxation. Whatever slight difference between these two curves there is, it may possibly be ascribed to differences in protein hydration.

4.3. The "outer-sphere" relaxation

The "D_2O"-curves, i.e., the "outer-sphere" relaxation rates, merit comment in spite of the fact that no quantitative structural parameter can be derived. It is necessary first to determine the pure paramagnetic contribution to these relaxation rates. It is usually done by subtracting the rates measured for the diamagnetic carbonmonoxyhaemoprotein, a contribution which is otherwise also inherently included in the measurements by "D_2O" experiments with paramagnetic haemoproteins.

The relaxation rates of the carbonmonoxyhaemoglobin and myoglobin solutions are reproduced in figs. 2 and 3, together with the curves obtained by subtracting these CO-curves from the "D_2O"-data, b – (c, d) and (b–c) respectively. The latter curves represent the "outer-sphere" relaxation due only to the rate induced by the paramagnetic haem-iron.

The diamagnetic rates are normalized per haem, and the rate ratio Mb/Hb is about four. This may be due to the fact that the monomeric myoglobin has a larger specific area of the protein surface exposed for interaction with solvent protons than the tetrameric haemoglobin. However, the remaining paramagnetic contribution to the "outer-sphere" relaxation of fluorometmyoglobin does not differ from that of fluoromethaemoglobin within the experimental error (20% to 10% from low to high temperature limits). Thus, in a qualitative way, which is the only possible one in discussing the "outer-sphere" relaxation for haemoproteins, one may conclude that the accessibility of the haem-pocket for the solvent molecules is very similar for fluorometmyoglobin and fluoromethaemoglobin from –15°C to +10°C.

5. Conclusions

A marker method has been developed by which one can distinguish between the mechanism of (i) "outer-sphere" relaxation, i.e., in the absence of any contribution to the rates from the relaxing species that may exchange between the site(s) of close association with the paramagnetic centre and bulk solvent, and (ii) "fast-exchange" by which the exchange contribution dominates over that of the "outer-sphere" relaxation.

The results obtained with this method eliminate the interpretation according to which the temperature enhancement of the relaxation rates in methaemoprotein solutions is due to the changes in the relative position of the haem-iron to the protein surface. They also strongly support the only other known interpretation, that of the thermally activated exchange rate. Hence, all those cases for haemoprotein solutions where such a temperature enhancement of PMR rates is observed may be discussed from the point of view of the energy of activation for the exchange mechanism as a parameter in defining the dynamics of the haem-pocket.

In the cases of established "fast-exchange" relaxation, i.e., for solutions of horse fluorometmyoglobin and human haemoglobin, and for aquomethaemoglobin of Chironomus, the site from which the protons exchange with bulk solvent from the haem-pocket, is at a distance greater than that defined by the sixth ligand site in the first coordination sphere of the haem-iron. This means that the PMR relaxation rate induced by these exchanging proton(s) may be useful as a parameter to describe the conformational state(s) of the haem-pocket in different haemoproteins and under different conditions.

The described marker method may also be applied in studies of the active sites of those enzymes activated by paramagnetic metal ions, or spin-labelled, for which an involved shape, like the haem-pocket, might be expected to exist.

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