INTERACTIONS OF SOLVENT WITH THE HEME REGION OF METHEMOGLOBIN AND FLUORO-METHEMOGLOBIN

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ABSTRACT It is now more than 20 years since Davidson and collaborators (1957, *Biochim. Biophys. Acta.* 26:370–373; *J. Mol. Biol.* 1:190–191) applied the theoretical ideas of Bloembergen et al. (1948. *Phys. Rev.* 73:679–712) on outer sphere magnetic relaxation of solvent protons to studies of solutions of methemoglobin. From then on, there has been debate regarding the relative contributions to paramagnetic solvent proton relaxation by inner sphere (ligand-exchange) effects and by outer sphere (diffusional) effects in methemoglobin solutions. Gupta and Mildvan (1975. *J. Biol. Chem.* 250:146–253) extended the early measurements, attributed the relatively small paramagnetic effects to exchange with solvent of the water ligand of the heme-Fe^{3+} ion, and interpreted their data to indicate cooperativity and an alkaline Bohr effect in the presence of inositol hexaphosphate. They neglected the earlier discussions entirely, and made no reference to outer sphere effects. We have measured the relaxation rate of solvent protons as a function of magnetic field for solutions of methemoglobin, under a variety of conditions of pH and temperature, and have given careful consideration to the relatively large diamagnetic corrections that are necessary by making analogous measurements on oxyhemoglobin, carbonmonoxyhemoglobin, and cyano- and azide-methemoglobin. (The latter two, because of their short electronic relaxation times, behave as though diamagnetic.) We show that the paramagnetic contribution to solvent relaxation can be dominated by outer sphere effects, a result implying that many conclusions, including those of Gupta and Mildvan, require reexamination. Finally, we present data for fluoro-methemoglobin, which relaxes solvent protons an order of magnitude better than does methemoglobin. Here one has a startling breakdown of the dogma that has been the basis for interpreting many ligand-replacement studies; in contrast to the prevailing view that replacement of a water ligand of a protein-bound paramagnetic ion by another ligand should decrease relaxation rates, replacement of H_2O by F^- increases the relaxation rate drastically. The data can all be reconciled, however, with what is anticipated from knowledge of ligand interactions in the heme region.

INTRODUCTION

In his classic paper on the theory of nuclear induction, Bloch (1946), concerned that proton spin-lattice relaxation times might be too long for convenient observation, noted that "it is recommendable, in this case, to add to the substance a certain percentage of paramagnetic atoms or molecules." Indeed, in an accompanying paper describing the first observation of a proton resonance in water (Bloch et al., 1946), the spin-lattice relaxation time T_1 of water

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protons in a "concentrated solution of $Fe(NO_3)_3$" was reported to be of the order of 10^{-4} to 10^{-5} s, some five orders of magnitude shorter than T_1 for protons in pure water.

Shortly thereafter, Bloembergen et al. (1948) developed a quantitative theory of solvent proton relaxation in pure water due to intramolecular proton-proton magnetic dipolar interactions. They also formulated an approximate theory of proton relaxation due to intermolecular proton-proton interactions arising from translational diffusion, which they then extended to account for proton relaxation by paramagnetic solute ions. The latter is a theory of "outer sphere" relaxation of protons by paramagnetic ions; contributions to relaxation from the formation of hydrated ions were not considered. The outer sphere contribution to the proton relaxation rate depends on the distance of closest approach of protons and paramagnetic ions, and on a correlation time determined by the relative diffusional motion of water molecules and ions. However, as Bloembergen et al. (1948) note: "It will be interesting to examine nuclear relaxation under conditions for which the characteristic time τ_C due to thermal motion is quite long. The actual τ_C may then be determined by the paramagnetic relaxation effect." This was a reference to the possibility that fluctuations in the orientation of the spin of the paramagnetic ion could determine the correlation time for the ion-proton outer sphere interaction.

Davidson and Gold (1957) and Kon and Davidson (1959) were among the first to apply the ideas of outer sphere relaxation of Bloembergen et al. (1948) to studies of solutions of macromolecules. They measured relaxation rates of solvent protons, at one value of magnetic field and temperature, in solutions of methemoglobin and metmyoglobin and applied the theory of outer sphere relaxation to obtain an estimate of the depth of the Fe^{3+}-ions below the surface of the protein. It was implicitly assumed that there was no contribution to the relaxation from exchange with solvent of water ligands in the first coordination sphere of the Fe^{3+}-ions; i.e., "inner sphere" effects were not considered. This issue was subsequently addressed by Wishnia (1960), who compared the relaxation contribution and temperature, again at one value of magnetic field, of Fe^{3+}-ions free in solution, in methemoglobin, and in conalbumin, (a protein of 76,000 daltons that contains two Fe^{3+}-ions per molecule which, from earlier studies, were thought to be close to the surface of the protein and readily accessible to solvent). Indeed, the paramagnetic relaxivity of conalbumin (i.e., the contribution to the solvent proton relaxation rate per mole of paramagnetic ion) was found to be about equal to that of the Fe^{3+}-aquoion, and about a factor 30 greater than that of methemoglobin at the particular magnetic field used. By this time, there was general agreement that proton relaxation in solutions of paramagnetic aquo-ions was dominated, in general, by inner sphere processes and ligand exchange (cf. Nolle and Morgan, 1957), and Wishnia (1960) interpreted his results for conalbumin as inner sphere interactions, and for methemoglobin as outer sphere interactions. However, he recognized that these assignments were somewhat equivocal, and suggested that a quantitative understanding of the experimental results in terms of the theories of the several mechanisms of relaxation required measurements as a function of temperature as well as an extension of such measurements to lower values of magnetic field.

Lumry et al. (1961) extended the hemoglobin experiments of Wishnia (1960) to include hemin and myoglobin, both ferrous and ferric, and concluded that the paramagnetic contributions to solvent proton relaxation by both metmyoglobin and methemoglobin were due to inner sphere relaxation, the latter conclusion being at variance with the interpretation

of similar data by Wishnia (1960). Lumry et al. (1961) were also among the first to report that the diamagnetic protein (in this case, oxyhemoglobin) contributed measurably to solvent proton relaxation as well, and to associate the effect with retardation of the motion of water molecules in the hydration shell of the protein molecules.

Thus, from the start, there was uncertainty regarding the relative contributions of inner sphere or ligand-exchange effects and outer sphere or diffusional effects in solutions of heme proteins; how to make the distinction when interpreting experimental results was a problem then, and is to this day. It is the situation regarding methemoglobin and fluoro-methemoglobin that we address in the present work, for reasons that follow.

Almost a quarter century ago, Davidson and Gold (1957) noted that "methemoglobin is less effective than Fe^{3+}-aquoion by a factor of about 100 in relaxing solvent water protons;" and "... it is probable that the heme group is not on the surface of the protein ...; it is reasonable to assert that the paramagnetic iron atom is 5–10 Å below the surface." Kon and Davidson (1959), who repeated the hemoglobin measurements and extended the studies to myoglobin, noted of the earlier work the "perceptible but relatively small relaxing effect of methemoglobin on water protons," indicated that "... the heme group is ... effectively shielded from solvent by the protein. The heme group may be in a crevice but we cannot say ... whether the relaxation is mainly due to a direct magnetic interaction with outer [sphere] water or ... with crevice water, followed by diffusion-exchange of crevice and outer water."

The theory of outer sphere relaxation as a function of magnetic field strength (without complex formation) was improved substantially in the early 1960's by Pfeifer (1961, 1962, 1963). The results, though straightforward to interpret physically, are rather complex algebraically. The physical picture is: when a solvent proton diffuses sufficiently close to a paramagnetic ion, the fractional change in separation of the two in a given time is relatively large, and the fluctuations in the local magnetic field seen by the proton are dominated by the diffusive motion. When the two are relatively far apart, the fluctuations in local field are dominated by the magnetic relaxation rate τ_S of the paramagnetic ion. It is the interplay of these two mechanisms that complicates the theory. The theory has two adjustable parameters, τ_S and d, the distance of closest approach of proton and ion; it also contains the diffusion constant of the solvent nuclei.

Hausser and Noack (1965) applied the results of Pfeifer to the study of the magnetic field dependence (dispersion) of the relaxation of water protons by dissolved O_2, and in so doing, restated the theoretical results (however, marred by a nontrivial typographical error), and gave an extensive set of (correct) graphical solutions. The results were simplified by Koenig and Schillinger (1969), who were concerned with relaxation of solvent protons by Fe^{3+}-ions in transferrin. They compared the contributions to the relaxivity to be expected from outer and inner sphere relaxation processes, suggested an appropriate geometric correction factor to be applied because of the restriction on access of solvent to the ion that is imposed by the protein itself when the paramagnetic ion is buried a distance d below the surface of the protein molecules, and gave the results in graphical form. Their results, for the limit of low fields, are reproduced in Fig. 1 for two values of τ_S in the range appropriate for methemoglobin. What should be clear is that when low-field (corresponding to proton Larmor frequencies below ~0.1 MHz) relaxivities are less than $\sim 10^3$ $M^{-1}s^{-1}$, corresponding to high field (say, 10–100 MHz) relaxivities of about 300 $M^{-1}s^{-1}$, the likelihood is that part, if not all, of the relaxation

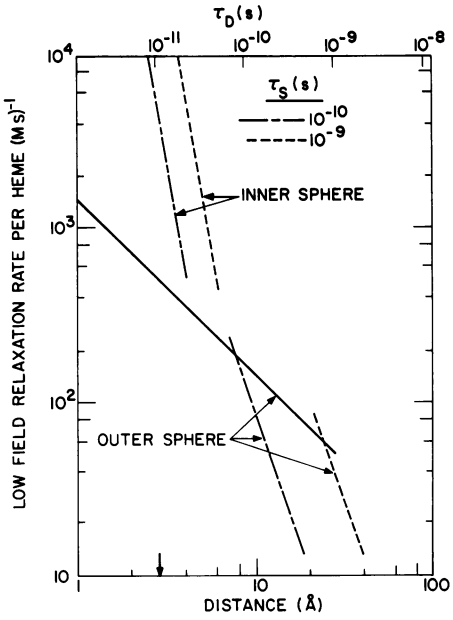

FIGURE 1 Relaxation rate of solvent water protons in solutions of proteins containing high spin Fe^{3+}-heme moities. For the lines marked outer sphere, it is assumed that the ion is buried a distance below the protein surface given by the abscissa, and inaccessible to solvent. The outer sphere contribution is computed for two values of τ_S, the relaxation time of the paramagnetism of the heme-iron, and is sensitive to its value for deeply buried ions. The inner sphere contribution to the relaxation is also shown, assuming one water molecule in rapid exchange, as a function of Fe^{3+}-proton separation. The arrow at 2.8 Å indicates the Fe^{3+}-proton separation for a water molecule ligand of the heme-iron. The results are computed for the limit of zero external magnetic field, and include a factor to account for geometric effects (see text).

can arise from outer sphere effects. The high-field relaxivity for methemoglobin solutions, given first by Davidson and Gold (1957) and remeasured and corrected by Kon and Davidson (1959), is (250 ± 50) s^{-1}/mol heme. These authors, as remarked above, ascribed all the relaxation to outer sphere effects. Pifat et al. (1973), in an extensive investigation of the temperature dependence of proton relaxation in solutions of methemoglobin, also concluded "... that the water molecule directly coordinated to the heme-iron ... is not exchanging with bulk solvent fast enough to affect the proton relaxation."

Gupta and Mildvan (1975) reported studies of the relaxation of solvent water protons by methemoglobin, as a function of temperature, pH, and magnetic field (but limited to relatively high values of magnetic field, 24–220 MHz), without considering outer sphere processes in the interpretation of any of their results. Despite the extensive history described above, they ascribed all the relaxation effects, including changes upon addition of inositol hexaphosphate, to inner sphere, exchange-limited, interactions of protons in the sixth ligand of the heme-iron. In addition, they measured the relaxation of solvent protons by fluoro-methemoglobin. Here, though F^- displaces the water ligand of methemoglobin, the relaxivity is an order of magnitude greater, which Gupta and Mildvan (1975) ascribe to relaxation of the proton of the distal histidine of the heme, and subsequent base-catalyzed exchange of this proton. This mechanism was proposed, notwithstanding the fact that it was shown by Koenig

and Schillinger (1969) (and reiterated several times since in relation to similar problems in carbonic anhydrase [Koenig and Brown, 1972; Koenig et al., 1974]) that base-catalyzed exchange near neutral pH cannot produce proton transfer to solvent at a rate sufficient to account for relaxation rates as large as found for fluoro-methemoglobin solutions.

In what follows, we present data on the dispersion (i.e., magnetic field-dependence) of solvent proton spin-lattice relaxation in solutions of methemoglobin, fluoro-methemoglobin and carbonmonoxyhemoglobin, (the latter data are used to correct for the diamagnetic contribution to the relaxation so as to obtain the paramagnetic contributions), and compare the results with theory. What is new is that the data are obtained over a wide range of magnetic fields, including very low values. This allows the results to be apportioned, in a unique fashion, to inner and outer sphere effects. We have done this over a range of values of temperature and pH for methemoglobin. We find a pH-dependence of both the inner and outer sphere contributions to the relaxivity, with a pK_a substantially below that of the acid-alkaline transition of methemoglobin near pH 8.1. For fluoromethemoglobin, the data require a rapidly exchanging water molecule in the heme pocket with one proton about a hydrogen-bond distance from the heme-bound F^--ion. The results will be put into context in what follows.

MATERIALS AND METHODS

Protein Preparation

Hemoglobin, in some cases, was prepared in the usual manner (Drabkin, 1946) from acid-citrate-dextrose treated blood obtained from the Greater New York Blood Bank. Additional details can be found in Lindstrom et al. (1976). In other cases, it was a gift from Dr. R. Nagel of the Albert Einstein College of Medicine. All samples were stripped of organic phosphate, mainly for ease of comparison with earlier data (Lindstrom and Koenig, 1974).

Relaxation Measurements

Only the spin-lattice relaxation rates of solvent protons are reported. Though the apparatus has not been described in detail, various aspects of the procedures, including limitations on accuracy and reproducibility, have been described (cf. Hallenga and Koenig, 1976, and references therein) and need not be repeated here.

Theory and Data Reduction

Interpretation of the data requires some care since the paramagnetic effects are often small, comparable in magnitude to the diamagnetic ones, and there is no assurance that the two contributions are independent, i.e., strictly additive. Recent work (Koenig et al., 1978) has shown that about half the diamagnetic relaxation of solvent protons in oxyhemoglobin arises from cross-relaxation interactions between solvent and protein protons; i.e., the solvent relation rates depend on the relaxation rates of the protein protons. These rates in turn can be affected by the spin-state of the heme-iron moities of the protein molecules. In particular, the relatively long paramagnetic relaxation time of Fe^{3+}-ions in methemoglobin may well alter the diamagnetic contribution to solvent proton relaxation; we have no way of ascertaining the magnitude of this potential correction. Rather, noting that protons in solutions of oxy-, carbonmonoxy-, deoxy-, azidemet-, and cyanomet-hemoglobin all have the same relaxation rates per mole heme, we assume that this value (for a given pH, field, temperature, etc.) is appropriate to subtract from the measured values to obtain the paramagnetic contribution to solvent proton relaxation rates.

The following formulas hold for R_0, the outer sphere relaxation in $M^{-1}s^{-1}$ due to Fe^{3+}-ions with S =

5/2 (cf. Koenig and Brown, 1973):

$$R_0(\tau_S/\tau_D \rightarrow \infty) = f_D(8.72 \times 10^{15}\, \tau_D/d^3)\, [0.7F(\Omega_S) + 0.3F(\Omega_I)]$$
$$\text{where } \Omega_{S,I} \equiv (6\omega_{S,I}\tau_D)^{1/2} \tag{1}$$

and

$$R_0(\tau_S/\tau_D \rightarrow 0) = f_S(7.27 \times 10^{15}\, \tau_S/d^3)\, [0.7J(\omega_S\tau_S) + 0.3J(\omega_I\tau_S)]$$
$$\text{where } J(\omega_{S,I}\tau_S) = (1 + (\omega_{S,I}\tau_S)^2)^{-1} \tag{2}$$

Here d, the distance of closest approach of solvent to Fe^{3+}-ion, is to be inserted in units of Angstroms. The diffusive correlation time is $\tau_D = d^2/3D$, where d is in cm, and D is the diffusion constant of solvent. F is a function very much like the Lorentzian J, but broader, and decreases to half its low field value of unity at $\Omega \simeq 1.3$. The factors f_D and f_S are geometric factors that are equal to unity if the ions are at the center of the sphere; if the buried paramagnetic ions, on the other hand, are regarded as a distance d beneath a semi-infinite plane (considering the protein surface as flat), then $f_D = 1/8$ (Koenig and Schillinger, 1969), and f_S can readily be calculated to be $1/6$.

Once the paramagnetic part of the relaxivity is obtained, it is straightforward to resolve it into an inner sphere, exchange-limited part and an outer sphere part, despite simplifying assumptions in the theory, such as neglecting ligand-field splittings of the levels of the paramagnetic ions, which (in the present case) are large compared to the Zeeman energies. This is so, because the high-field (the range 20–100 MHz in the present systems) outer sphere relaxation rate must decrease to 0.3 of its low-field value because of the symmetry of a liquid solution. By contrast, any exchange-limited inner sphere contribution, because the relaxities are as low as they are, must be independent of magnetic field-strength. These two facts allow a unique separation of the paramagnetic contribution into inner and outer sphere contributions; one simply subtracts a field-independent relaxation rate from the data such that the remaining contribution drops to 0.3 of its low-field value at high field.

As has been argued earlier in a related situation (Koenig and Schillinger, 1969), the various levels arising from the ligand field effects are equally populated in our temperature range, so that a diffusing proton will see an average local magnetic field which, if the averaging is sufficiently rapid, will be identical to that which would be observed in the absence of ligand field effects. Such is most likely the case here, so that the theory of outer sphere relaxation as given here should be quite accurate.

RESULTS

Fig. 2 A shows data for methemoglobin and carbonmonoxyhemoglobin at 6°C, and a comparison of the experimental results with the theory of outer sphere relaxation. The respective contributions to the solvent proton relaxation rate of buffer, diamagnetic effects, inner sphere (exchange-limited) paramagnetic effects, and outer sphere paramagnetic effects are shown. Fig. 2 B shows analogous results at 35°C. The values for the parameters derived from the Theory are in Table I.

Data were also taken on samples of azide- and cyano-methemoglobin; the results confirm earlier indications (Fabry and Reich, 1966; Gupta and Mildvan, 1975) that these solutions of low- and zero-spin forms of hemoglobin have the same relaxation dispersions, with no paramagnetic contribution.

The value $1/8$ was used throughout for the geometric factor, Eqs. 1 and 2, even though the samples are not in the limit $\tau_S/\tau_D \rightarrow \infty$. The proper value is between $1/8$ and $1/6$, and depends upon knowing the ratio τ_S/τ_D very well. This in turn depends upon knowing the shape of the paramagnetic relaxation dispersion curve to high accuracy, as may be seen in Fig. 3. Here the

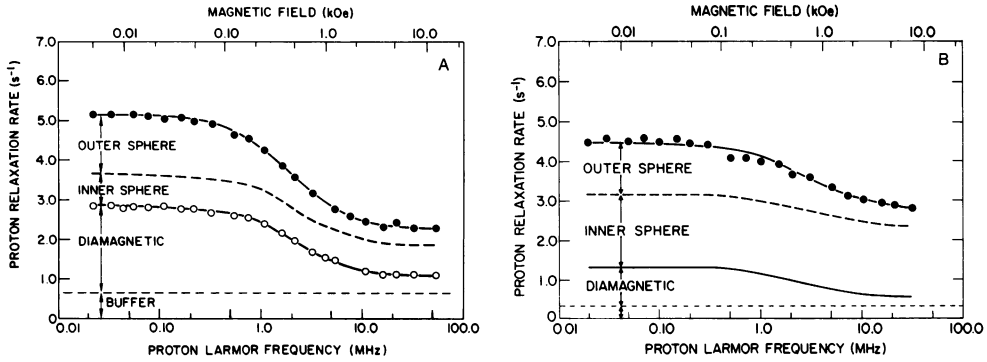

FIGURE 2 (*A*) Magnetic field dependence of the solvent proton spin-lattice relaxation rate for methemoglobin (●), and carbonmonoxyhemoglobin (○) at 6°C. The methemoglobin was 1.67 mM protein in 0.2 M phosphate buffer, pH 6.25. The carbonmonoxyhemoglobin concentration was 1.5 mM protein, pH 6.09; these data were scaled linearly to the concentration of the methemoglobin before plotting. The solid line through the methemoglobin data results from a least-squares comparison of the difference between the two sets of data with the theory of outer sphere relaxation. The contributions to the relaxation rates of the buffer, (diamagnetic) carbonmonoxyhemoglobin, exchange-limited inner sphere, and outer sphere effects are all indicated. (*B*) Analogous to (*A*), but for 35°C.

paramagnetic relaxation contribution is taken from Fig. 2 A, along with the best fit, and two other fits corresponding to the limits of no diffusion and no paramagnetic relaxation. The value of d derived from the latter fit was used to obtain the τ_S value for the former. The results are in Table I. The point is that, given uncertainties in the diamagnetic corrections as well as uncertainties in the measurements of relaxation rates, concentrations, etc., a precise value for the ratio τ_S/τ_D is difficult to deduce from the data. Nonetheless, the resolution of the results into inner and outer sphere contributions (indicated by the horizontal lines, Fig. 3) is quite definitive. Additionally, using 1/8 for the geometric factor underestimates, possibly substantially, the importance of outer sphere relaxation.

Fig. 4 results from data comparable to that in Fig. 2 A for seven higher values of pH. The outer sphere contributions at both low and high fields are shown, together with a least-squares fit of two titration curves, each with a single titrating proton with a common value for the pK_a.

TABLE I
VALUES FOR THE PARAMETERS OF THE THEORIES OF RELAXATION OBTAINED FROM LEAST-SQUARES COMPARISONS WITH THE DATA

Sample	Figure	T	pH	d or r	τ_D	τ_S	τ_M
		°C		Å	*ps*	*ns*	*μs*
Met—Hgbn	2*A*	6	6.25	7.6	170	1.0	170
Met—Hgbn	4	6	7.78	9.6	270	1.2	250
Met—Hgbn	2*B*	35	6.3	5.3	33	1.8	65
Met—Hgbn	3	6	6.25	9.6	270		160
Met—Hgbn	3	6	6.25	9.6		0.12	180
Fluoro—Met—Hgbn	5	6	6.6	3.1–3.7*		0.06	≤0.05

*Ignoring ligand field splittings gives the upper limit of the range; including them in an approximate way gives the lower limit.

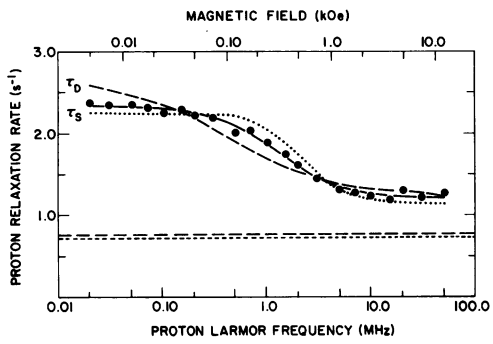

FIGURE 3

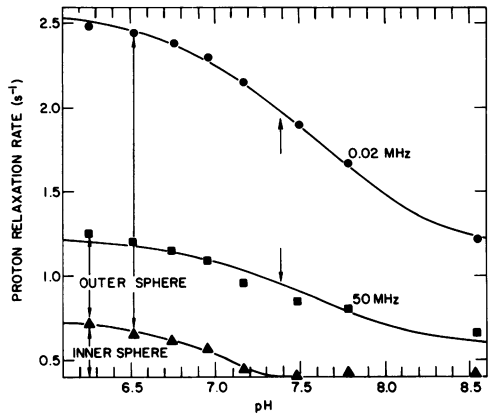

FIGURE 4

FIGURE 3 A comparison of the best fit to the paramagnetic contribution to the relaxation rates of Fig. 2 *A* (●) assuming that only τ_D is important (— — —), and that only τ_s is important (· · ·). The partitioning of the data into inner sphere (horizontal lines) and outer sphere components is seen to be insensitive to the ratio τ_s/τ_D.

FIGURE 4 Inner and outer sphere contributions to the relaxation rate of solvent protons as a function of pH at 6°C. The outer sphere contributions are shown for two values of magnetic field, corresponding to proton Larmor frequencies of 0.02 and 50 MHz; the original data are shown by (●) and (■), respectively. The field-independent inner sphere contribution is indicated by (▲). The results were obtained from data and procedures analogous to those for Fig. 2 *A*, for each value of pH indicated. The outer sphere contributions at both fields were fit, by a least-squares criteria, to a single titration with the same pK_a (7.38) for both curves. The solid lines through the data points result from this fit.

The smaller, less accurately known, inner sphere contribution is also shown. The important conclusion, though qualitative, is that access of solvent, as solvent, to the center of the heme groups (as measured by *d*) decreases significantly as pH is increased. Concomitant with this, the inner sphere protons (water molecules) leave at a slower rate as pH is increased. Additionally, as has been noted previously (Fabry et al., 1971), the pK_a, 7.53 under the present conditions, is significantly <8.1, the known value for the ionization of the sixth-ligand water of the heme-iron.

In Fig. 5, solvent proton relaxation rates for solutions of fluoro-methemoglobin and methemoglobin are compared. The samples are at relatively low pH, as indicated, and the concentration of fluoride ion, ~150 mM, was determined to be sufficient to saturate the relaxation effects at this pH. The solid line through the fluoro-methemoglobin data result from a least-squares comparison of the paramagnetic part of the relaxation with the usual theory with the usual simplifications, the most serious in the present case being the neglect of effects due to ligand-field splittings. The results, included in Table I, are $\tau_S = 6.0 \times 10^{-10}$ s; a short exchange time, no greater than 5×10^{-8} s; and 3.7 Å for the proton-Fe^{3+} separation, assuming one proton being relaxed at a time. This value will be reduced by the factor $(105/38)^{1/6}$ to 3.1 Å when one makes the grossest corrections demanded by the ligand-field effects (Koenig et al., 1971; Koenig, 1978; Koenig and Brown, 1980; Sternlicht, 1965; Lindner, 1965).

Gupta and Mildvan (1975) present relaxation data for fluoro-methemoglobin solutions at

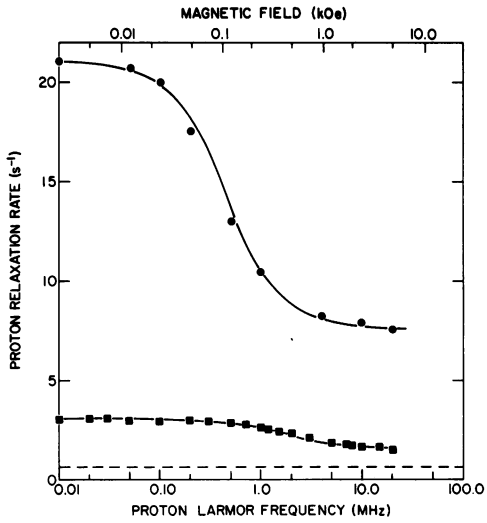

FIGURE 5 Magnetic field dependence of the solvent proton spin-lattice relaxation rate for a solution of fluoro-methemoglobin (●), and a comparable sample of methemoglobin (■) at 6°C. The fluoro-methemoglobin is 1.0 mM protein in 0.05 M *bis*-Tris, 0.1 M NaCl, 0.1 M NaF buffer, pH 6.55. The methemoglobin sample, except for the NaF, is comparable. The relaxation contribution of the protein-free buffer is indicated by the dashed horizontal line. The solid line through the fluoro-methemoglobin data results from a least-squares comparison of the data with the usual theory of inner sphere relaxation; the results, assuming one exchanging proton, are 3.7 Å for the proton-Fe^{3+} separation (without corrections for ligand fields splitting), $\tau_S = 6.0 \times 10^{-10}$ s, and an upper limit of about 5×10^{-8} s for the resident lifetime of this proton.

four values of field from 24 to 220 MHz. Their data agree well with ours at 20 MHz (the temperature dependence is small; cf., their Fig. 2), and disperse again (as expected) at a higher field corresponding to a correlation time of 9.6×10^{-10} s at 23°C, in quite good agreement with our value of 6.0×10^{-10} s at 6°C. Their derived distance, 4.11 ± 0.03 Å, is at variance with ours given their stated limits of error of <1% but, as indicated, the uncertainties of the theory contribute uncertainties of order 30% and do not justify these limits.

DISCUSSION

Methemoglobin

The major result is that outer sphere relaxation accounts for a substantial part of the observed solvent proton relaxation rate. At low fields and 6°C, it is about two-thirds of the total at all values of pH considered; at 35°C it is less, consistent with a contribution from thermally activated exchange of a water molecule from the inner sphere (Pifat et al., 1973). Moreover, as mentioned, these are lower limits. Though there is no way of separating the two contributions experimentally unless measurements are made at both low and high fields, as is done here (and as we are uniquely equipped to do), the theory of outer sphere relaxation is sufficiently developed and tested so that one can readily calculate contribution to be expected from outer sphere effects; they will clearly be significant, if not dominant, in methemoglobin solutions.

Additionally, as small as the inner sphere contribution to the relaxivity is, it is still too large by almost an order of magnitude to be accounted for by acid-base catalyzed proton exchange (as contrasted with exchange of entire water molecules), a point discussed by Koenig and Schillinger (1969) and in more depth by Koenig and Brown (1973).

Despite this background, Gupta and Mildvan (1975) ascribe all the relaxation of solvent protons in solutions of methemoglobin to an inner sphere exchange process mediated by acid-base catalyzed proton exchange from the sixth ligand of the heme-iron, and quote uncertainties of 15% for the proton off-rate, derived using this invalid assumption. They also measure proton-deuteron isotope effects, and observe significant and interesting changes in relaxation rates upon addition of inositol hexaphosphate (IHP). Their conclusions must all be reexamined, in light of the present work. It must be demonstrated, for example, whether IHP alters the inner or outer sphere contributions; only then can one speculate on models and mechanisms, and derive values for model parameters.

With regard to the pH-dependence of the paramagnetic contribution to the relaxation, it is clear (Fig. 4) that much of the pH-dependence is in the outer sphere contribution. The source of the pK_a for this dependence (7.4 in the present case) is unknown. As pointed out previously (Fabry et al., 1971), this pK_a is well below that of the ionization of the sixth ligand water of the heme (about 8.1), with its associated optical transition and susceptibility change, contrary to the identification made by Gupta and Mildvan (1975; cf. Table V). It should be noted here that the titrations, Fig. 4, show no indication of this acid-alkaline transition at high values of pH, contrary to expectation, but it could well be that associated with the transition to alkaline methemoglobin is the appearance of an exchangeable water molecule in the heme pocket, much as in the case of fluoro-methemoglobin (see below), that propitiously balances the expected decrease in relaxation rates over the limited pH range for which data are shown.

Fluoro-methemoglobin

The tertiary structures of fluoro-methemoglobin and methemoglobin are almost identical, the quaternary structures are identical, and the heme-iron atoms are essentially all high spin in both cases. The major differences are that the sixth ligand water is replaced by fluoride (with no contribution to the x-ray-difference map), and a peak in the map appears that suggests a water molecule with its oxygen 2.8 Å from the fluoride ion and 4.3 Å from the heme center (Deatherage et al., 1976). This corresponds to one water proton 3.4 Å from the heme center, assuming a linear fluoride-proton hydrogen bond (which turns out to be 1.9 Å), and the second water proton about 5 Å from the heme center, a distance sufficiently great so that its magnetic relaxation rate may be ignored compared to the first, to first order.

These difference peaks "probably arise from water molecules stabilized in the ligand pockets by hydrogen bonding to the negatively charged fluoride ions. . ." (Deatherage et al., 1976). Asher et al. (1977), and Asher and Schuster (1979), find peaks in the resonance Raman spectra of fluoro-methemoglobin that they attribute to a heme-fluoride stretch with the fluoride ions hydrogen bonded to the water molecules discussed by Deatherage et al. (1976). These water molecules, we presume, are responsible for the observed solvent relaxation in fluoro-methemoglobin. The separation of the proton and heme-iron as estimated from the x-ray data (3.4 Å) is consistent with the range 3.1–3.7 Å obtained above from the relaxation data, given the uncertainties introduced by ligand field effects. By contrast,

relaxation of an exchangeable proton of the distal histidine as a mechanism for relaxation of solvent protons, as suggested by Gupta and Mildvan (1975) cannot explain the observed relaxation since once again the previous arguments regarding the maximum rate of exchange of such protons by acid-base catalysis apply. On the other hand, hydrogen-bonded water molecules can readily exchange in times $\sim 10^{-8}$ s, the upper limit suggested by the fit to the data in Fig. 5. (Note simply that the dielectric relaxation of solvent water, which requires the breaking of two hydrogen bonds, has a relaxation time of $\sim 10^{-11}$ s.)

Summary

Whenever the contribution of a strongly paramagnetic, protein-bound ion to the spin-lattice magnetic relaxation rate of solvent protons is no greater than, say, 500 $(M\ s)^{-1}$, one must consider that the relaxation may be due entirely, or substantially, to long range magnetic interactions between an inaccessible, buried paramagnetic ion and the solvent protons as they diffuse in the vicinity of the protein. This is outer sphere relaxation. It is difficult to resolve small relaxation effects into an outer sphere part, and an inner sphere contribution due to slowly exchanging protons but a lower limit for the outer sphere contribution may be readily obtained. For solutions of methemoglobin, such an analysis (as presented here) has not been done in the past. The present analysis shows that attributions made by earlier workers are incorrect and many conclusions based on these attributions must be reinterpreted.

For solutions of fluoro-methemoglobin, the situation is quite different. The solvent relaxation rates are large, despite the fact that there is no solvent ligand of the heme-iron, due to the presence of an additional water molecule in the heme pocket (not present in methemoglobin) that is indicated by x-ray difference maps and resonance Raman spectroscopy.

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