Measurement of Heme Accessibility in Soybean Ferric Leghemoglobin *a* and Its Complexes by Proton Magnetic Relaxation†

David L. Ollis, Peter E. Wright,* James M. Pope, and Cyril A. Appleby

ABSTRACT: The accessibility of the heme and solvent-exchange dynamics in solutions of soybean ferric leghemoglobin *a* and several of its complexes have been determined by measurement of the relaxation rates of water, acetone, and methanol protons. Acetone and methanol were used at low concentrations as probes of outer-sphere and inner-sphere exchange processes, respectively. Water proton relaxation in solutions of ferric leghemoglobin is exchanged limited. The rate of exchange of water molecules or protons from the environment of the heme is greater than that for ferric myoglobin and ferric hemoglobin. Methanol binds to ferric leghemoglobin in a homogeneous first-order reaction and in competition with fluoride, a known iron ligand. Both optical and NMR measurements indicate binding of methanol to the sixth coordination position of the iron atom. Methanol proton relaxation by both ferric leghemoglobin and whale ferric myoglobin is exchange limited. Exchange is more rapid for leghemoglobin (4.0×10^3 s^{-1} at 25 °C) than for myoglobin (9.5×10^2 s^{-1} at 25 °C). Relaxation of acetone protons occurs by fast outer-sphere exchange from the heme pocket. Acetone is thus a useful probe of heme accessibility. Acetone proton relaxation rates show the heme to be more accessible in ferric leghemoglobin than in ferric myoglobin. Conformational changes occur on binding of fluoride or acetate which decreases the accessibility of the heme in leghemoglobin. In myoglobin, binding of fluoride or formate appears to make the heme more accessible to acetone molecules. These results indicate that the heme in soybean ferric leghemoglobin is in a flexible region of the protein and is more accessible to solvent than in vertebrate myoglobins and hemoglobins. The relevance of this open, flexible heme pocket to the physiological O_2-binding reaction of ferrous leghemoglobin is discussed.

Water proton relaxation enhancement (PRE)[1] measurements in solutions of ferric heme proteins have been widely used to probe the immediate environment of the heme and its accessibility to solvent molecules. Interpretation of the data is complex, however, since relaxation of protons of the bulk solvent may arise from inner-sphere (exchange of water molecules or protons from the coordination sphere of the ferric ion) or outer-sphere (exchange of noncoordinated water molecules or protons from the vicinity of the metal ion) exchange processes. A stereochemical marker method, in which the relaxation of the aliphatic protons of alcohols in the heme protein solutions is measured, has been proposed to distinguish between these relaxation mechanisms (Vuk-Pavlović et al., 1974). However, at the high concentrations of alcohols used by these workers there is considerable risk of conformational perturbation of the protein. Our own unpublished experiments show many heme proteins to be sensitive to the presence of even moderate concentrations of organic solvents. Binding of methanol and ethanol to ferric myoglobin and ferric hemoglobin has recently been reported (Brill et al., 1976). Reactions of peroxidase with hydrogen peroxide or cyanide are inhibited by ethanol, and it has been suggested that this alcohol binds to the sixth coordination position of the heme (Dunford & Hewson, 1977). In view of the evidence for interactions between alcohols and heme proteins, we have used a modification of the stereochemical marker method (Vuk-Pavlović et al., 1974) together with conventional water PRE measurements in studies of proton relaxation in solutions of heme proteins. The present method differs from the stereochemical marker method in that only low, nonperturbing concentrations of the probe molecules are used. In addition, two types of probe are used: acetone, as a probe of outer-sphere exchange processes and of heme accessibility, and methanol as a probe of inner-sphere exchange processes. This method should have general utility in the study of heme proteins by nuclear relaxation.

Leghemoglobins are monomeric heme proteins found in *Rhizobium*-infected nitrogen-fixing legume root nodules. They appear to facilitate the diffusion of oxygen to the nitrogen-fixing bacteroids (Appleby, 1974). The three-dimensional structure of lupin ferric leghemoglobin has been determined to 2.8-Å resolution and is very similar to that of ferric myoglobin (Vainshtein et al., 1978). However leghemoglobins differ from myoglobins in many of their properties, such as high oxygen affinity (Wittenberg et al., 1972), magnetic properties (Ehrenberg & Ellfolk, 1963; Appleby et al., 1976), and ability to bind bulky ligands (Ellfolk, 1961; Appleby et al., 1973). The structural parameters which give rise to these characteristic properties of leghemoglobin are not yet apparent from the three-dimensional structure. We have therefore undertaken detailed studies by NMR methods to probe the heme pocket structure and dynamics of leghemoglobin. In the present paper, we discuss the heme accessibility in ferric leghemoglobin and some of its complexes, the dynamics of solvent exchange, and the mechanisms of solvent proton relaxation.

Materials and Methods

Ferric leghemoglobin *a* was extracted from soybean root nodules and purified by a previously described procedure (Appleby et al., 1975). Whale ferric myoglobin was purchased from Sigma Chemical Co. and used without further purification. The concentrations of solutions used for NMR experiments were determined spectrophotometrically by using $a_{403} = 157$ mM^{-1} cm^{-1} for dilutions of ferric leghemoglobin

† From the School of Chemistry, University of Sydney, Sydney, N.S.W. 2006, Australia (D.L.O. and P.E.W.), the School of Physics, University of New South Wales, Kensington, N.S.W. 2033, Australia (J.M.P.) and the Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, A.C.T. 2601, Australia (C.A.A.). *Received March 14*, 1980; *revised manuscript received August 26, 1980*. This work was supported by the Australian Research Grants Committee.

[1] Abbreviations used: PRE, proton relaxation enhancement; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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in 100 mM Mes buffer, pH 5.2, or by the pyridine hemochrome method (Ohlsson & Paul, 1976). pH was determined by using a Radiometer pH meter 26 fitted with an Ingold microelectrode. For the pH titration, unbuffered solutions of leghemoglobin (4 mM) containing NaCl (10 mM) were used. The pH was varied by mixing solutions of protein at high and low pH rather than by direct titration with acid or base.

(Carbonmonoxy)leghemoglobin was prepared by a previously described method (Wright & Appleby, 1977). The cyanide, nicotinate, fluoride, acetate, and formate complexes were prepared from the ferric protein by titration with the appropriate ligands. Reagents were of analytical grade, and glass-distilled water was used throughout. All reagents used in the NMR experiments were treated, if necessary, with Dowex A-1 to remove any paramagnetic metal ion impurities.

Water proton relaxation times were measured with a Bruker SXP pulsed NMR spectrometer at frequencies from 5 to 60 MHz. To obtain adequate signals at frequencies lower than 10 MHz, it was necessary to use rather concentrated solutions of ferric leghemoglobin (5.35 mM). Longitudinal relaxation times (T_1) were measured by using a 180°–τ–90° pulse sequence. The null point method was used at 20 MHz. At other frequencies values of T_1 were obtained by plotting log ($M_\infty - M_\tau$) vs. τ, where M_∞ is the equilibrium value of the magnetization and M_τ is that at a time τ after the 180° pulse. Longitudinal relaxation times of methanol and acetone protons were measured at 90 MHz with a Bruker HX90 spectrometer. Values of T_1 were determined from plots of log ($M_\infty - M_\tau$) vs. τ. Leghemoglobin solutions in D_2O were prepared by dialysis. The protein concentration was 0.15 mM for methanol and 1 mM for acetone relaxation measurements.

The water proton relaxation rates were observed to be equal within experimental error in solutions of diamagnetic ferrous (carbonmonoxy)leghemoglobin and equimolar solutions of the ferric leghemoglobin nicotinate or cyanide complexes. The absence of a measurable paramagnetic contribution to the relaxation rate in solutions of the nicotinate or cyanide complexes is attributed to the very short electron-spin relaxation time of the low-spin ferric iron, $T_{1e} \leq 2 \times 10^{-12}$ s (Wüthrich, 1970). These complexes can thus be used instead of (carbonmonoxy)leghemoglobin to measure the diamagnetic contribution of the protein to the proton relaxation. The paramagnetic contributions to the relaxation rates were calculated as the difference between the relaxation rates in solutions of leghemoglobin and those measured in equimolar solutions of the carbon monoxide, nicotinate, or cyanide complexes.

Results

Temperature Dependence of the Water Proton Relaxation Rates. The observed temperature dependence of the paramagnetic contribution to the water proton molar relaxation rates of solutions of ferric leghemoglobin and its fluoride complex is shown in Figure 1. For leghemoglobin fluoride the values of $1/T_{1p}$ decrease with increasing temperature. The Arrhenius plot of Figure 1 is linear within experimental error and is consistent with fast exchange of water molecules or protons out of the paramagnetic environment. In solutions of ferric leghemoglobin $1/T_{1p}$ was observed to increase with increasing temperature over the measured temperature range (Figure 1). It is known from optical (Ehrenberg & Ellfolk, 1963) and EPR measurements (Appleby et al., 1976) that the population of the high-spin state in ferric soybean leghemoglobin *a* increases with increasing temperature, and this might be expected to result in enhanced solvent proton relaxation. However, it has been shown by optical difference spectroscopy (C. A. Appleby, unpublished observations) that

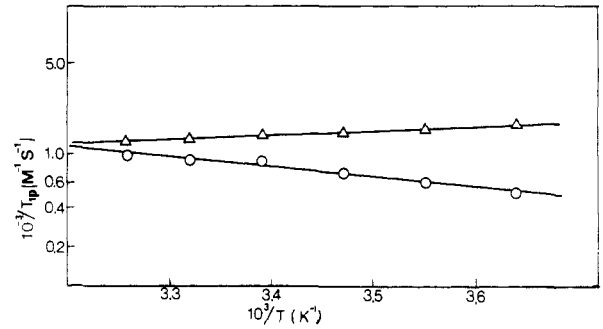


FIGURE 1: Temperature dependence of paramagnetic contribution to molar longitudinal water proton relaxation rates measured at 20 MHz in solutions of ferric soybean leghemoglobin (4.0 mM) in phosphate buffer (100 mM, pH 7.0). (○) Ferric leghemoglobin; (△) fluoride (150 mM).

at pH 6.0 there is only a ~5% increase in the high-spin component from 0 to 25 °C. This change is clearly too small to account for the 75% increase in $1/T_{1p}$ over the same temperature range. The negative slope of the Arrhenius plot in Figure 1 thus indicates that relaxation is limited by chemical exchange.

As a result of the spin-state equilibrium, soybean ferric leghemoglobin is only ~70% high spin at room temperature (C. A. Appleby, unpublished observations). The measured water proton relaxation rate must thus be corrected for the "dilution" effect of the low-spin component. Since electron-spin relaxation is very rapid for low-spin ferric hemes (Wüthrich, 1970), the ~30% low-spin leghemoglobin will not contribute to the paramagnetic relaxation. The value of $1/T_{1p}$ for water protons normalized to "100% high-spin" ferric leghemoglobin is then 1.4×10^3 s^{-1} M^{-1} at 22 °C.

If chemical exchange occurs between the first coordination sphere of a metal ion and the bulk solvent, the paramagnetic contribution to the net longitudinal relaxation rate is given by (Luz & Meiboom, 1964; Swift & Connick, 1962)

$$\frac{1}{T_{1p}} = \frac{pq}{T_{1M} + \tau_M} \quad (1)$$

where p is the ratio of the concentration of the paramagnetic ion to the concentration of the ligands, q is the number of ligands in the coordination sphere, T_{1M} is the relaxation time of the bound nuclei, and τ_M is their mean residence time at the paramagnetic center. For water proton relaxation, eq 1 becomes

$$\frac{1}{T_{1p}} = \frac{Nq}{55.6} \frac{1}{T_{1M} + \tau_M} \quad (2)$$

where N is the concentration of the paramagnetic ion. Assuming relaxation in ferric leghemoglobin solutions occurs by exchange of entire water molecules from the first coordination sphere of the heme iron atom (see Discussion), the rate of exchange ($1/\tau_M$) with bulk solvent (calculated by using eq 2) is 7.8×10^4 s^{-1} at 22 °C.

Effects of Acetone and Methanol on Leghemoglobin. Spectrophotometric methods were used to investigate the interaction of acetone and methanol with soybean ferric leghemoglobin. Acetone (12 mM) had no detectable effect on the optical spectrum of ferric leghemoglobin (100 μM) in phosphate buffer (pH 6.0) at 20 °C. Thus, under the conditions of the NMR experiments (12-fold excess of acetone over 1 mM leghemoglobin), binding of acetone, insofar as it may be monitored by changes in the absorption spectrum, is negligibly small. At higher concentrations of acetone, an inhomogeneous binding reaction was observed which perturbs

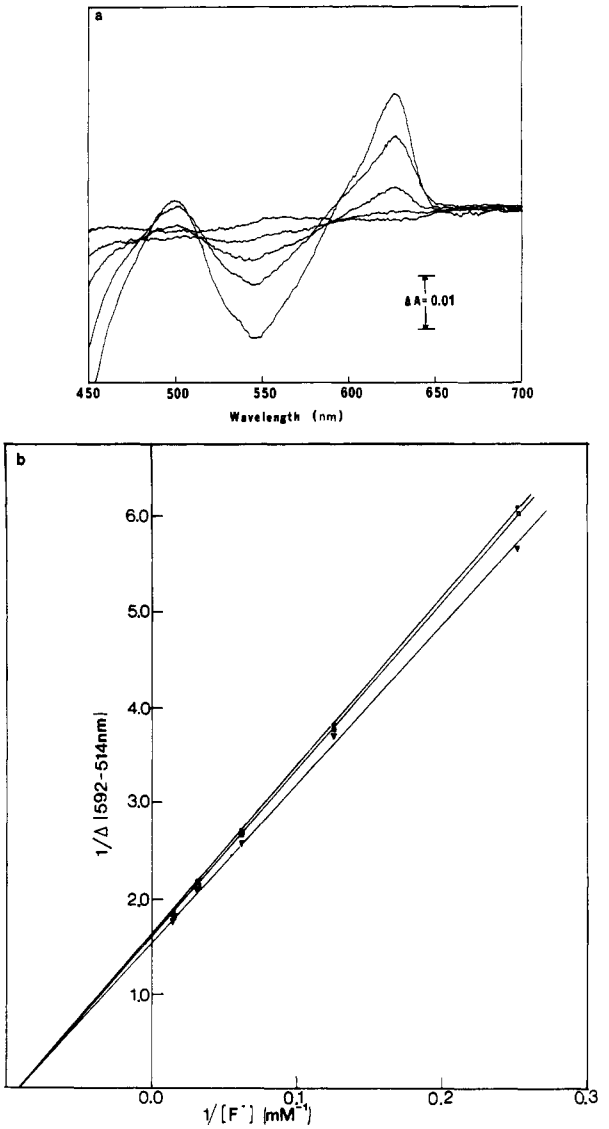


FIGURE 2: (a) Titration of ferric leghemoglobin with acetone by optical difference spectroscopy. The initial concentration of leghemoglobin was 111 μM in potassium phosphate buffer (100 mM, pH 6.0) at 20 °C. Increments of aqueous acetone (to 0, 25, 100, 250, and 500 mM nominal concentration) were added to the sample cuvette and equal volumes of water to the reference cuvette (both 10-mm light path). Difference spectra were recorded in a Cary 14R spectrophotometer fitted with 0–0.1-A slide-wire. (b) Double-reciprocal plots obtained by optical difference spectroscopy for titrations of ferric leghemoglobin (100 μM) with fluoride in the presence of varying concentrations of acetone [(▽) 0 mM; (□) 20 mM; (○) 500 mM] in potassium phosphate buffer (100 mM, pH 6.0) at 20 °C. The wavelength pair, 592–514 nm, was chosen to indicate leghemoglobin fluoride formation with minimal interference from the conformational change induced by acetone.

the spin-state equilibrium of ferric leghemoglobin (Figure 2a). The reaction leads to a greater proportion of high-spin ferric heme, as is evident from the increase in the high-spin peak at 626 nm and the decrease in the low-spin band at 546 nm on addition of acetone. No isosbestic points are observed (Figure 2a) and the curvature evident in a double-reciprocal plot [(absorbance change)$^{-1}$ vs. [acetone]$^{-1}$] (not shown) shows that the apparent binding affinity increases rapidly at higher acetone concentration. It is clear that this is not a simple first-order binding reaction but probably reflects a protein conformational change.

Fluoride is known to bind to the heme iron atom of leghemoglobin (Appleby et al., 1976). Spectrophotometric titrations of ferric leghemoglobin with fluoride in the presence of varying concentrations of acetone produced a series of linear double-reciprocal plots which intercept on the horizontal axis (Figure 2b). This demonstration of noncompetitive binding (Dixon & Webb, 1964) provides evidence that acetone is not directly coordinated to the heme iron atom of leghemoglobin. Additional evidence comes from NMR which shows a relatively small paramagnetic contribution to the acetone proton relaxation rate (Table II).

When ferric leghemoglobin is titrated with aqueous methanol at 25 °C, a spectrophotometrically detectable reaction occurs (Figure 3a). The increase in absorbance at 626 nm and the decrease at 546 nm indicate a transition to a high-spin ferric species. The well-defined isosbestic points at 509 and 594 nm and linear double-reciprocal plot ($A_{626-547nm}^{-1}$ vs. [methanol]$^{-1}$) (not shown) suggest a homogeneous first-order reaction but do not identify the methanol binding site. The dissociation constant, K_{diss}, of the methanol–leghemoglobin complex is estimated to be 280 mM.

Spectrophotometric titrations of ferric leghemoglobin with fluoride in the presence of varying concentrations of methanol produced a series of linear double-reciprocal plots which intercept on the vertical axis (Figure 3b). This demonstration of competitive binding (Dixon & Webb, 1964) strongly suggests that methanol, like fluoride, binds directly to the iron atom.

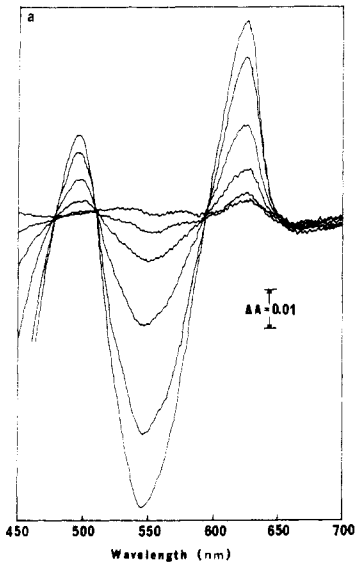
Temperature Dependence of Acetone Proton Relaxation Rates. The temperature dependence of the longitudinal proton relaxation rates of acetone (11.4 mM) in solutions of ferric leghemoglobin (1 mM) in phosphate buffer (100 mM, pH 6.0) in D_2O is shown in Figure 4. Since acetone is not an iron ligand, we conclude from the observed decrease in $1/T_1$ with increasing temperature that it is in fast outer-sphere exchange from the heme pocket. That the heme pocket is in fact accessible to acetone is indicated by the much smaller paramagnetic contribution to proton relaxation for *tert*-butyl alcohol, a bulky molecule which is less likely to penetrate the heme pocket ($1/T_{1p} = 0.05$ s^{-1} for 12 mM *tert*-butyl alcohol in 1.5 mM ferric leghemoglobin compared to $1/T_{1p} = 0.49$ s^{-1} for 12 mM acetone in a 1 mM solution of the protein).

Temperature Dependence of Methanol Proton Relaxation Rates. The observed increase in methanol proton relaxation rates with increasing temperature (Figure 5) in solutions of ferric leghemoglobin shows that relaxation is limited by chemical exchange. The paramagnetic contribution to the longitudinal relaxation rate of methanol protons is given by a modification of eq 1

$$\frac{1}{T_{1p}} = \frac{f_M}{T_{1M} + \tau_M} \tag{3}$$

where f_M is the mole fraction of methanol complexed to the paramagnetic species. f_M can be obtained from the dissociation constant of the methanol–leghemoglobin complex. The exchange rate ($1/\tau_M$) of methanol out of the environment of the paramagnetic center can be calculated by using eq 3 and is 4.0×10^3 s^{-1} at 25 °C.

Under conditions of exchange-limited relaxation, only an upper limit on the distance of approach of methanol protons to the iron atom can be obtained. Such a calculation is useful, however, to ascertain whether the measured relaxation times are consistent with exchange of coordinated methanol. Because of the complexities introduced by the anisotropic g values of high-spin ferric proteins, we have carried out the reverse procedure, i.e., estimated the dipolar contribution to longitudinal relaxation rate ($1/T_{1M}$) of coordinated methanol by using the Solomon–Bloembergen equation (Solomon, 1955; Bloem-



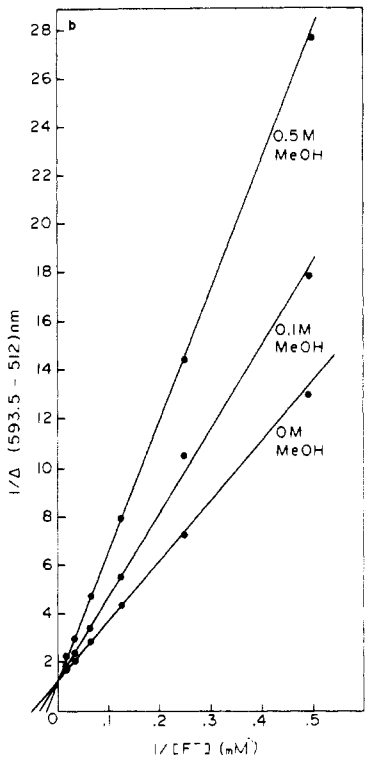


FIGURE 3: (a) Titration of ferric leghemoglobin with methanol by optical difference spectroscopy. The initial concentration of leghemoglobin was 116 μM in potassium phosphate buffer (100 mM, pH 7.0) at 25 °C. The procedure was as described for Figure 2. Nominal methanol concentrations were 0, 11, 37, 111, 370, and 1111 mM. (b) Double-reciprocal plot obtained by optical difference spectroscopy for titration of ferric leghemoglobin (100 μM) with fluoride in the presence of varying concentrations of methanol in potassium phosphate buffer (10 mM, pH 7.0) at 25 °C. The wavelength pair, 593.5–512 nm, was chosen to indicate leghemoglobin fluoride formation with minimal interference from formation of the leghemoglobin–methanol complex.

bergen, 1957) modified for g-factor anisotropy (Sternlicht, 1965)

$$\frac{1}{T_{1M}} = \frac{2}{30}\frac{\gamma_I^2\beta^2 S(S+1)}{r^6}\left[\frac{1}{3}(g_\parallel^2 + 2g_\perp^2) + g_\parallel^2\cos^2\chi + g_\perp^2\sin^2\chi\right]\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right) \quad (4)$$

where r is the distance between the electron and nuclear spins, ω_I and ω_S are the nuclear and electronic Larmor precession

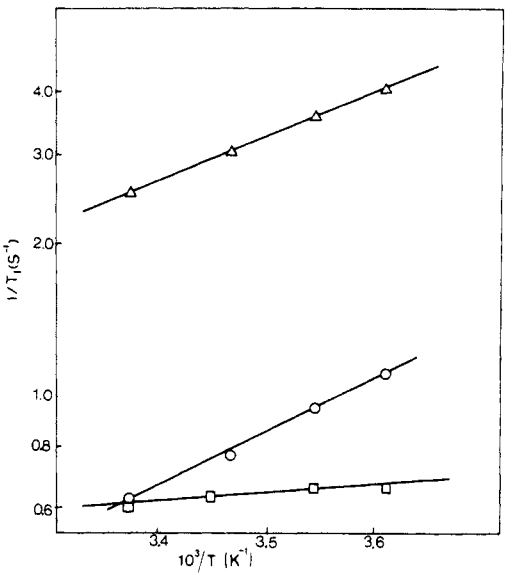


FIGURE 4: Temperature dependence of acetone proton longitudinal relaxation rates measured at 90 MHz in solutions of ferric leghemoglobin (1.0 mM) and ferric myoglobin (1.0 mM) in phosphate buffer (100 mM, pH 6.0) in D_2O. The concentration of acetone was 11.4 mM. (O) Ferric leghemoglobin; (Δ) ferric leghemoglobin plus fluoride (200 mM); (□) whale ferric myoglobin.

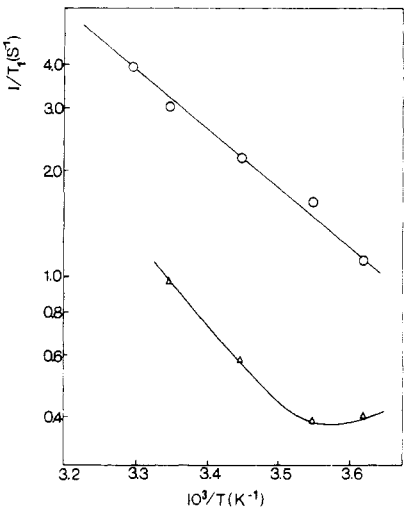


FIGURE 5: Temperature dependence of methanol proton longitudinal relaxation rates measured at 90 MHz in solutions of (O) ferric leghemoglobin (0.15 mM) and (Δ) ferric myoglobin (0.15 mM) in phosphate buffer (100 mM, pH 6.0) in D_2O. The concentration of methanol was 8 mM.

frequencies, τ_c is the correlation time that modulates the electron spin–nuclear spin dipolar interaction, χ is the angle between r and the magnetic symmetry axis, and the other symbols have their usual meanings (Dwek, 1973). For relaxation by high-spin ferric heme proteins the dominant correlation time is the electron-spin relaxation time, τ_s (Gupta & Mildvan, 1975; Hershberg & Chance, 1975). Assuming an upper limit on the Fe–O distance of 2.5 Å for a bound methanol molecule and taking $\tau_c = 10^{-10}$ s (see later discussion), we estimated $1/T_{1M}$ to be greater than $\sim 2 \times 10^4$ s^{-1} for the methyl group protons. The experimentally determined lower limit on $1/T_{1M}$ ($1/T_{1M} > 4.0 \times 10^3$ s^{-1}) is clearly not inconsistent with exchange of a methanol ligand. Both the NMR and optical measurements are thus in accord with binding of methanol to and exchange from the sixth coordination position of the heme iron atom.

pH Dependence of Water Proton Relaxation Rates. The pH dependence of the water proton molar longitudinal re-

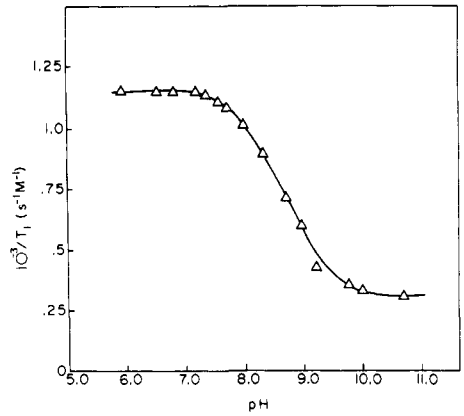


FIGURE 6: The pH dependence of the molar water proton longitudinal relaxation rates measured at 20 MHz and 22 °C in unbuffered solutions of ferric leghemoglobin (4 mM) in 10 mM NaCl.

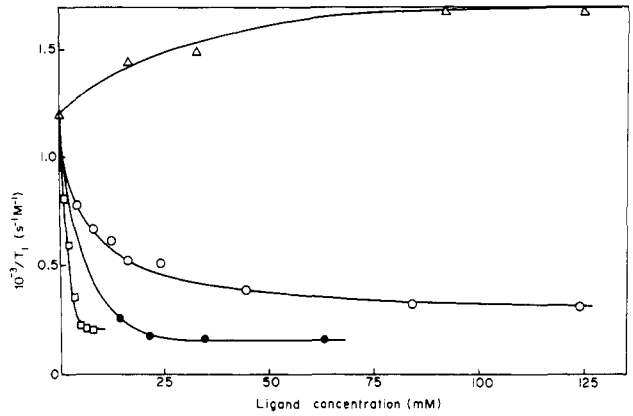


FIGURE 7: Effect of ligands on molar water proton longitudinal relaxation rates measured at 20 MHz and 22 °C in solutions of ferric leghemoglobin in phosphate buffer (100 mM, pH 7.0). (△) Fluoride; (○) acetate; (●) formate; (□) nicotinate.

laxation rate is shown in Figure 6. The decrease in relaxation rate with increasing pH is consistent with a transition to a low-spin form of leghemoglobin. The observed pK of 8.7 agrees within experimental error with the value of 8.34 determined by spectrophotometry and ascribed to formation of leghemoglobin hydroxide (Ellfolk, 1961). The NMR and optical methods clearly monitor the same ionization process. A plot of log [Y/(1 − Y)] vs. pH, where Y is the fraction of the low-spin (high-pH) form, has a slope of unity indicating that a single proton is involved in the alkaline transition.

Titration with Ligands. The effect of various iron ligands on the longitudinal water proton relaxation rates in solutions of leghemoglobin was measured and is summarized in Figure 7 and Table I. To gain further insight into the mechanism of the relaxation processes for these leghemoglobin complexes, we also measured relaxation rates of methanol and acetone protons under conditions such that the protein was fully ligated (Table II).

Acetone and Methanol Proton Relaxation in Solutions of Myoglobin Complexes. Longitudinal relaxation times were measured for acetone and methanol protons in solutions of whale ferric myoglobin and certain of its complexes. The results are summarized in Table II. The observed temperature dependence of the acetone proton relaxation rate (Figure 4) is consistent with a fast outer-sphere relaxation mechanism. However, ferric myoglobin is seen to be less efficient than ferric leghemoglobin in relaxing acetone protons. The observed increase with increasing temperature of the relaxation rates of the methyl group protons of methanol in solutions of ferric myoglobin (Figure 5) indicates exchange-limited relaxation,

Table I: Water Proton Relaxation Rates in Solutions of Leghemoglobin[a]

complex[b]	$1/T_1$ (s^{-1} M^{-1})	$1/T_{1p}$ (s^{-1} M^{-1})[c]
ferric leghemoglobin	1150	977,[d] 1.4×10^3[e]
fluoride	1700	1527
acetate	810	637
formate	310	137
azide	173	0
pyridine	152	0
nicotinate	200	27
ferrous (carbonmonoxy)-leghemoglobin	173	

[a] Measured at 20 MHz and 22 °C on solutions of leghemoglobin (4 mM) in phosphate buffer (100 mM, pH 7.0). [b] In all cases, sufficient ligand was added to fully form the complexes. [c] Paramagnetic contribution to the relaxation, calculated by subtraction of $1/T_1$ for the diamagnetic ferrous (carbonmonoxy)leghemoglobin. [d] Experimental value of $1/T_{1p}$. [e] $1/T_{1p}$ normalized for 100% high-spin ferric leghemoglobin (see text).

Table II: Acetone and Methanol Proton Relaxation in Solutions of Ferric Leghemoglobin and Ferric Myoglobin Complexes[a]

	$1/T_{1p}$ (s^{-1})			
	leghemoglobin		myoglobin	
complex	acetone[b]	methanol[c]	acetone[b]	methanol[c]
aquo	0.49,[d] 0.70[e]	2.1	0.49	0.80
fluoride	2.38	2.15	3.45	
acetate	0.28	0.45	0.59	
formate	0.21	0.09	0.85	
nicotinate	0	0		
cyanide			0	0

[a] Measured at 90 MHz and 25 °C in phosphate buffer (100 mM, pH 6.0). [b] Protein, 1.0 mM; acetone, 12 mM. [c] Protein, 0.15 mM; methanol 8 mM. [d] Experimental value of $1/T_{1p}$. [e] $1/T_{1p}$ normalized for 100% high-spin ferric leghemoglobin (see text).

Table III: Methanol and Water Proton Exchange Rates in Heme Proteins

protein	$1/\tau_M$ for water protons (s^{-1})	$1/\tau_M$ for methanol (s^{-1})
ferric hemoglobin	5.6×10^3 [a]	
ferric myoglobin	1.4×10^4 [a]	9.5×10^2 [b]
ferric leghemoglobin	7.8×10^4 [c]	4.0×10^3 [b]

[a] Rate of exchange of entire water molecules at 25 °C (Maričić et al., 1966). [b] Rate of exchange of methanol molecules at 25 °C (this work). [c] Rate of exchange of entire water molecules at 22 °C (this work).

as for ferric leghemoglobin. The overall relaxation rate is notably slower in myoglobin solutions, however. The dissociation constant for the methanol complex with myoglobin is 170 mM at 25 °C (Brill et al., 1976). The rate of exchange of a methanol ligand from the heme in myoglobin (calculated by using eq 3) is 9.5×10^2 s^{-1} at 25 °C, which is slower than for leghemoglobin (Table III).

Frequency Dependence of Water Proton Relaxation Rate in Ferric Leghemoglobin Solutions. Direct comparison of heme accessibility in ferric leghemoglobin and ferric myoglobin from measurements of nuclear relaxation rates requires information on the relative electron-spin relaxation rates of the high-spin ferric heme in these proteins. The frequency dependence of the water proton relaxation rate in solutions of ferric leghemoglobin was thus measured. The relaxation rate is frequency independent from 20 to 60 MHz. The onset of

dispersion is observed at frequencies lower than ~20 MHz, but the magnitude is small since only the relatively small contribution to the overall relaxation from fast outer-sphere exchange of water molecules will be frequency dependent. The observed relaxation dispersion for ferric leghemoglobin is exactly comparable with that reported for ferric myoglobin (Lanir, 1977). No attempt was made to calculate the electron-spin relaxation time from these data since the average distance of approach to the iron atom of water molecules in the outer sphere is needed and is not known. Whatever its exact magnitude, however, it is clear that τ_s must be very similar in soybean ferric leghemoglobin and in ferric myoglobin. For myoglobin, estimates of τ_s vary between 6×10^{-11} s from the NMR dispersion measurements (Lanir, 1977) and 2×10^{-10} s from EPR line widths (Fabry & Eisenstadt, 1974; Asakura et al., 1972).

The relative electron-spin relaxation times in the fluoride, acetate, and formate complexes of ferric leghemoglobin and ferric myoglobin were determined by measuring the widths of hyperfine shifted resonances in their 270-MHz ^{1}H NMR spectra. The widths at half-height of the resolved heme methyl group resonances in the spectra of the fluoride complexes of these two heme proteins are similar (approximately 450–500 Hz at 25 °C). We thus expect the electron-spin relaxation times to be similar. Acetate binds only weakly to ferric myoglobin, but formate forms a high-spin complex with heme methyl group resonances at similar chemical shifts (Morishima et al., 1977) to those of leghemoglobin acetate (Trewhella et al., 1979). The widths of these resonances in myoglobin formate (230–300 Hz at 25 °C) and in leghemoglobin acetate (~230 Hz) indicate comparable electron-spin relaxation times. Line widths are considerably greater in the spectra of the fluoride complexes, consistent with slower electron-spin relaxation.

Discussion

Water and Methanol Exchange Rates. The observed increase in water proton relaxation rate in solutions of soybean ferric leghemoglobin with increasing temperature (Figure 1) shows that relaxation is limited by exchange of water protons out of the environment of the paramagnetic heme. It is of note that even at temperatures approaching the freezing point, water proton relaxation is dominated by chemical exchange rather than outer-sphere relaxation processes. This is in marked contrast to water proton relaxation in solutions of ferric myoglobin (Mildvan et al., 1971; Vuk-Pavlović et al., 1974) and ferric hemoglobin (Maričić et al., 1966; Vuk-Pavlović et al., 1974) where outer-sphere exchange processes dominate at low temperatures. Clearly, water proton exchange is more facile in soybean ferric leghemoglobin.

The present water proton relaxation measurements provide no direct information as to whether exchange of entire water molecules or water protons occurs. Neither do they indicate directly whether exchange is from the first coordination sphere of the heme iron atom. Our experiments with methanol, however, strongly support coordination of this alcohol to the iron atom in ferric leghemoglobin. In view of the chemical similarity of water and methanol and the observation of a common exchange-limited proton relaxation mechanism (Figures 1 and 5), it seems most probable that the water proton relaxation experiments are monitoring exchange of water molecules or water protons from the coordination sphere. For the present we assume, for the purposes of comparison with other heme proteins, that relaxation in soybean ferric leghemoglobin solutions occurs by exchange of entire water molecules from the sixth coordination site of the heme. The rate calculated from the water proton relaxation data is 7.8×10^4 s^{-1} at 22 °C. This is considerably more rapid than exchange of water molecules from the heme pockets of ferric myoglobin or ferric hemoglobin for which rates of 1.4×10^4 s^{-1} and 5.6×10^3 s^{-1}, respectively, at 25 °C have been obtained (Table III). It is stressed that the overall conclusion that water proton exchange is more rapid from the heme pocket of leghemoglobin is valid irrespective of the mechanism of exchange. Further experiments are necessary to elucidate this mechanism.

Exchange of methanol from the first coordination sphere of the iron atom is also considerably faster for ferric leghemoglobin than for ferric myoglobin (Table III). Such facile ligand exchange might be expected if the heme pocket in leghemoglobin was more open and accessible than in the other heme proteins, but other factors such as differences in bonding to the iron atom cannot immediately be discounted.

Mechanism of Fluoride-Induced Relaxation Enhancement. Enhancement of both water and acetone proton relaxation rates occurs when fluoride is added to solutions of soybean ferric leghemoglobin (Figure 7, Table II). The observed decrease in $1/T_{1p}$ with increasing temperature (Figures 1 and 4) clearly indicates fast exchange of solvent molecules from the heme environment. Similar relaxation enhancement by fluoride has been reported previously for several other heme proteins. Various mechanisms have been proposed including (1) specific and fast exchange of a proton hydrogen bonded to the fluoride ligand (Fabry & Eisenstadt, 1974; Gupta & Mildvan, 1975), (2) a conformational change allowing greater heme accessibility in the fluoride complexes (Vuk-Pavlović, 1976), and (3) an increase in the electron-spin relaxation time on formation of the fluoride complex (Vuk-Pavlović & Benko, 1975). It is difficult to distinguish between these mechanisms from water proton relaxation data alone. However, the observation of acetone proton relaxation enhancement in leghemoglobin fluoride is inconsistent with mechanism 1. The observed relaxation enhancement clearly arises in part from formation of the fully high-spin fluoride complex from the mixed-spin ferric leghemoglobin. An additional factor which must be considered is the longer electron-spin relaxation times of the fluoride complexes of many heme proteins, as indicated by the line width of the room-temperature EPR spectrum (Asakura et al., 1972; Villafranca, 1976). Although this is probably the dominant mechanism, conformational changes in the heme pocket upon fluoride binding may also be important and will be discussed later.

It will be noted in Tables I and II that relaxation enhancement by fluoride is more pronounced for acetone protons than for methanol or water protons. For water and methanol, efficient exchange-limited relaxation from the first coordination sphere of the heme iron atom is replaced by equally efficient (fortuitously) relaxation by rapid exchange of water or methanol molecules from the immediate environment of the heme in fluoroleghemoglobin. Consequently, little relaxation enhancement is observed. For acetone, on the other hand, relaxation is by a fast outer-sphere exchange mechanism from the heme pocket of both ferric leghemoglobin and its fluoride complex and is very sensitive to changes in electron-spin relaxation rate.

Relaxation by Leghemoglobin Complexes. The relaxation rates of water, acetone, and methanol protons are all considerably reduced upon addition of acetate or formate to solutions of ferric leghemoglobin (Figure 7 and Tables I and II). The heme in the formate complex of leghemoglobin exists as an equilibrium mixture of high-spin and low-spin ferric states (Trewhella et al., 1979), and this is reflected in the pronounced

decrease in relaxivity. Acetate binds at the sixth coordination position of the iron atom in ferric leghemoglobin (Trewhella et al., 1979; Harutyunyan et al., 1978) to form a high-spin complex (Ehrenberg & Ellfolk, 1963; Appleby et al., 1976). The pronounced decrease in the relaxation rate of water and methanol protons probably arises from a change in the mechanism of relaxation due to blocking of the axial coordination site by a slowly exchanging acetate ligand. Changes in T_{1e} on formation of the acetate complex are unlikely.

Titration of ferric leghemoglobin with nicotinate, pyridine, or azide results in a decrease in water proton relaxation rate (Figure 7 and Table I). These ligands all form low-spin ferric complexes with leghemoglobin.

Heme Accessibility in Soybean Ferric Leghemoglobin and Its Complexes. The paramagnetic contribution to the proton relaxation rates of the outer sphere probe, acetone, in solutions of leghemoglobin and myoglobin (Table II) provides direct information on the relative accessibility of the high-spin ferric heme. NMR dispersion measurements show that the electron-spin relaxation time is comparable for these two proteins. Relaxation by ferric leghemoglobin is more efficient ($1/T_{1p}$ = 0.70 s^{-1}, corrected for the "dilution" effect of the low-spin component of the spin-state mixture) than in solutions of whale ferric myoglobin ($1/T_{1p}$ = 0.49 s^{-1}). The heme is clearly more accessible in soybean ferric leghemoglobin than in ferric myoglobin.

The magnitude of the fluoride-induced relaxation enhancement for acetone protons is much smaller for leghemoglobin (3-fold) than for myoglobin (7-fold) (Table II). Furthermore, the relaxation of acetone protons by myoglobin fluoride is overall more efficient than that due to leghemoglobin fluoride ($1/T_{1p}$ = 3.45 and 2.38 s^{-1}, respectively). Since the electron-spin relaxation rates do not appear to differ significantly, the acetone proton relaxation rates indicate differences in heme accessibility. On the basis of EPR line widths (Asakura et al., 1972; Villafranca, 1976), τ_s is ~5 times longer for myoglobin fluoride than for ferric myoglobin, which is consistent with the measured acetone proton relaxation enhancement. A slight increase in heme pocket accessibility on binding fluoride cannot, however, be discounted. For leghemoglobin, on the other hand, the much smaller relaxation enhancement and the lower paramagnetic relaxivity of the fluoride complex suggest a decrease in heme pocket accessibility on binding fluoride to the ferric protein.

The acetone proton relaxation measurements also provide evidence for conformational changes upon formation of carboxylate complexes of leghemoglobin and myoglobin. The relaxation rates of acetone protons in ferric myoglobin solutions are observed to increase upon formation of the formate complex (Table II). This relaxation enhancement most probably results from an increase in heme accessibility since EPR line width measurements indicate little change in the transverse electron-spin relaxation rate (Hershberg & Chance, 1975). We note that acetate also enhances the relaxivity of ferric myoglobin (Table II).

Since leghemoglobin acetate is a high-spin ferric heme complex, it may be compared with myoglobin formate in its ability to relax solvent protons. Whereas formate enhances the relaxivity of ferric myoglobin toward acetone, formation of the acetate complex of leghemoglobin considerably reduces the acetone proton relaxation rate (Table II). Since high-resolution NMR spectra show that the electron-spin relaxation times are comparable for these two complexes, these results indicate lower heme accessibility in leghemoglobin acetate than in myoglobin formate. It appears that binding of acetate (and presumably also formate) to the heme of leghemoglobin results in a conformational change which restricts access to the heme pocket.

A study of heme accessibility in *Lupinus luteus* leghemoglobin by solvent proton relaxation has been reported (Vuk-Pavlović et al., 1976). This showed that the heme is more accessible and its protein environment more flexible than is the case for vertebrate hemoglobins, in accord with the present results for soybean leghemoglobin. However, the relaxation properties of lupin leghemoglobin differ from those of the soybean protein in two aspects: (1) water protons were seen to be in fast exchange from the heme pocket of lupin leghemoglobin and (2) the frequency dispersion of the water proton relaxation rates differs markedly. No satisfactory explanation for these differences can yet be given. It appears that while the overall heme accessibility may be comparable in the leghemoglobins from lupin and soybean, there may be subtle differences in the structure of the proteins in the immediate vicinity of the heme.

The present proton relaxation measurements provide unequivocal evidence that the heme pocket in ferric soybean leghemoglobin (and probably in all leghemoglobins) is more open and accessible to solvent than in vertebrate hemoglobins and myoglobins. Binding of ligands such as fluoride and acetate (and probably also formate) appears to induce conformational changes which make the heme less accessible. [Further evidence for these ligand-induced conformational changes has been found by using high-resolution NMR spectroscopy (J. Trewhella and P. E. Wright, unpublished observations).] This unusually open and flexible heme pocket, if retained in the ferrous protein, provides a plausible explanation for the unique O_2-binding properties of leghemoglobin: the open and accessible heme pocket would facilitate reaction with O_2 and account for the very rapid on rate constant (Wittenberg et al., 1972; Imamura et al., 1972), while a subsequent conformational change resulting in closure of the heme pocket could stabilize the complex. Some experimental evidence is available for ferrous leghemoglobin which supports this mechanism. The ease with which the bulky ligand nicotinate binds to both ferrous and ferric leghemoglobin compared to myoglobin or hemoglobin suggests that the heme is more accessible in the deoxy and aquoferric forms of leghemoglobin (Peive et al., 1972; Appleby et al., 1973, 1976), while high-resolution NMR studies provide evidence that the heme pocket is closed in the O_2 and CO complexes (Wright & Appleby, 1977). More detailed NMR studies are in progress to provide information on the nature of the conformational changes involved in reactions with ligands and to further substantiate the proposed mechanism of the physiological oxygen-binding process.

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Circular Dichroism, Raman Spectroscopy, and Gel Filtration of Trapped Folding Intermediates of Ribonuclease†

Andrzej Gałat, Thomas E. Creighton, Richard C. Lord,* and Elkan R. Blout*

ABSTRACT: The intermediates of ribonuclease with one to four disulfide bonds trapped during refolding of the reduced protein have been compared to the fully reduced and native forms of the protein by gel filtration, circular dichroism, and Raman spectroscopy. Correctly refolded ribonuclease is indistinguishable from native protein, while a three-disulfide intermediate has a compact conformation which is very similar, but not identical. In contrast, all other intermediates with one to four disulfide bonds are qualitatively similar to fully reduced ribonuclease by their circular dichroism and Raman spectra, although the disulfide cross-links affect the dimensions of the polypeptide chain. The apparent absence of stable partially ordered structures in the initial disulfide intermediates implies that during refolding there are relatively few constraints on formation on disulfide bonds, although their formation is probably not entirely random. The stable conformation appears during refolding only when the three or four disulfide bonds capable of stabilizing a native-like conformation of the entire polypeptide chain occur simultaneously.

Bovine pancreatic ribonuclease A is one of the most extensively studied proteins [reviewed most recently by Richards & Wyckoff (1971)] and remains the classic subject of experimental studies of protein unfolding and refolding. There is still no general consensus as to the mechanism or pathway of the refolding.

Many studies have been made of unfolding and refolding with the four disulfide bonds kept intact. Most equilibrium measurements of the reversible unfolding transition produced by denaturants, high temperatures or pressures, extremes of pH, or combinations of these factors have indicated that the transition is cooperative, with essentially only the fully folded and fully unfolded states populated at equilibrium, all partially

† From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115 (A.G. and E.R.B.), MRC Laboratory of Molecular Biology, University Medical School, Hills Road, Cambridge CB2 2QH, United Kingdom (T.E.C.), and Spectroscopy Laboratory and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (A.G. and R.C.L.). *Received June 6, 1980.* This work was supported by the National Science Foundation (Grant CHE78-26217) and the U.S. Public Health Service (Grant AM07300).

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