SPIN RELAXATION OF IRON IN MIXED STATE HEMOPROTEINS

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ABSTRACT In hemoproteins the relaxation mechanism of iron is Orbach for high spin (HS) and Raman for low spin (LS). We found that in met-hemoglobin and met-myoglobin, under conditions in which the two spin states coexist, both the *HS* and the *LS* states relax to the lattice through Orbach-like processes. Also, very short (~1 ns) and temperature independent transverse relaxation times T_2 were estimated. This may result from the unusual electronic structure of mixed states hemoproteins that allows thermal equilibrium and interconversion of the spin states.

INTRODUCTION

The low temperature dependence of the spin lattice relaxation time T_1 of the iron in ferric hemoproteins has been extensively studied (1–4) and it is now well established that the relaxation mechanism depends on the spin of Fe^{3+}. In the high spin (*HS*) form the relaxation is dominated by an Orbach process (1) whose characteristic energy, Δ, is identified with the zero field splitting $2D$ of the $^6A_1$ state. In the low spin (*LS*) form a Raman process is the dominant relaxation mechanism. The unusual temperature dependence $1/T_1 \propto T^{6.3}$ observed in this case has been explained in terms of the fractal form of the protein (2–4).

In this letter we report on a new temperature dependence of the spin-lattice relaxation time T_1 of the LS state of the Fe^{3+} ion in ferric methemoglobin (hb) and metmyoglobin (mb) observed in powdered samples in which both the *LS* and *HS* states coexist (5,6). Also an estimate of the transverse relaxation time T_2 of the two spin states in hb and mb is given.

EXPERIMENTAL

The samples were prepared from lyophilized sperm whale mb (Sigma Chemical Co., St. Louis, MO) and from hb obtained by hemolysis of horse blood. The protein solutions were reduced with a 20-fold excess of ferricyanide followed by dialysis against the adequate buffer. These were a 0.1 M phosphate buffer pH 5.8 for mb and a 0.1 M sodium-potassium phosphate buffer pH 7.0 for hb. Samples were dried by fluxing N_2 gas through the protein solution.

The electron spin resonance (ESR) measurements were performed in the temperature range from 4.2K to 14K, using the continuous saturation technique with an X-band electron spin resonance spectrometer of conventional design. Two different cryostats: an Helitran (LTD-3-110; Air Products & Chemicals, Inc., Allentown, PA) for temperatures higher than 6K and a Wilmad dewar (WG-830; Wilmad Glass Co., Inc., Buena NJ) for 4.2K were utilized. The Air Products APD-E temperature controller was used to vary the temperature which was measured with two Au-Fe 0.07% vs. chromel thermocouples. One of those was located at 2 cm above the sample. Calibration curves which related the temperature of the heater and the sample were also obtained. The $g = 6$ and $g = 2$ ESR lines of the HS state and the $g = 2.25$ line of the LS state were monitored. The half saturation power, $P_{1/2}$, and the inhomogeneity parameter, b, were obtained by fitting the experimental data with the expression (7)

$$\frac{I}{\sqrt{P}} = \frac{I_{max}}{(1 + P/P_{1/2})^{b/2}}, \tag{1}$$

where P is the incident microwave power, I is the intensity of the derivative of the ESR absorption line and I_{max} is the limiting value of Eq. 1 at very low power. T_2 was estimated from the value of b and the line-width measurements. We used Castner's curves (8) (with b equal to a) to obtain the parameter $K(a) = \Delta H_{pp}/\Delta H_L$ which relates the observed peak to peak line width, ΔH_{pp}, to ΔH_L, the Lorentzian spin packet line width. T_2 was obtained from the expression

$$T_2 = \frac{2K(a)}{\sqrt{3}\gamma\Delta H_{pp}}, \tag{2}$$

where $\gamma = g\mu_B/\hbar$. Here μ_B is Bohr's magneton and $\hbar$ is Planck's constant. T_1 is calculated from the equation

$$T_1 = \frac{1}{\gamma^2 P_{1/2} T_2}. \tag{3}$$

The error in $K(a)$ due to the assumption that $b = a$ is at most a factor of two (8) which would increase the value of T_2 by a factor of two and decrease T_1 by the same factor. Since our conclusions deal mainly with the relative values of T_1 as a function of temperature, these uncertainties are not very significant.

RESULTS AND DISCUSSION

The complete spectra of met-mb and met-hb are shown in Fig. 1. They were both obtained at 170 mW Klystron power, which is sufficient to partially saturate the low spin spectra. The lines at $g = 6$ and $g = 1.99$ belong to the high spin state, all the other lines to the low spin state.

In order to get the correct ratios of the two states, one has to use low power conditions. Our data show that below 20K the high to low spin ratio in both proteins is practically

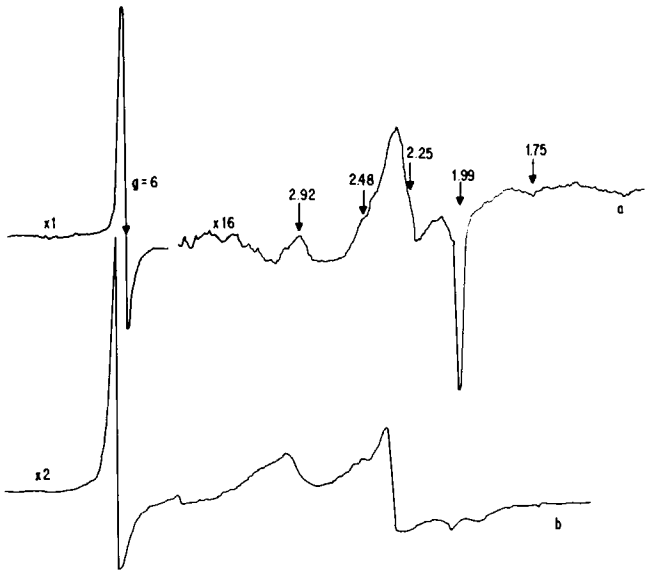

FIGURE 1 EPR spectra of: (*a*) met-mb at 8.5K, 170 mW power, (*b*) met-hb at 17K, 170 mW power. Notice that the amplification of the $g = 6$ line of mb is much smaller than that of the remaining lines.

temperature independent, but depends on the cooling cycle (5). For met-hb at a cooling rate of 30K/min that ratio is ~0.5, while for met-mb at 25K/min it is 1.7.

The parameter *b* varies only slightly with temperature. Its magnitude is 1.8 ± 0.4 for all samples at temperatures between 4K and 14K. This yields the values of T_2 given in Table I.

With the exception of the results reported in reference 9 for *HS* hb and mb where a very similar value for *b* (temperature independent between 3.5K and 20K) is obtained, we are not aware of other measurements of T_2 as a function of temperature in hemoglobin. Blum and Ohnishi (10) report $T_2 \simeq 10^{-8}$ s is ferricytochrome *c* between 6K and 25K; Pattison et al. (11) obtain $T_2 \simeq 10^{-6}$ s in met-mb for both spin states at 2.1K.

Typical results for met hb are shown in Fig. 2. Since we are only interested in the temperature dependence of the relaxation times and not in their absolute values, we have normalized the vertical scale for an overlap of the values of T_1 for *HS* and *LS*. This stresses the exponential behavior of the relaxation of both spin states. The actual values of T_1 at 10K are: $T_1 = 3.5 \times 10^{-7}$ s for *HS* with g = 6, $T_1 = 5 \times 10^{-6}$ s for *HS* with $g = 2$ and $T_1 = 5 \times 10^{-5}$ s for *LS* with g = 2.25, and the characteristic energy is $\Delta_{hb} = 14.5 \pm 1.7$ cm^{-1}. To facilitate a comparison with the temperature behavior of Raman processes a $T^{6.3}$ curve is also traced. In the case of met mb (Fig. 3), $\Delta_{mb} = 14.0 \pm 2.0$ cm^{-1} and the values of T_1 at 10K are: 3.0×10^{-7} s for *HS* with $g = 6$, 7.0×10^{-6} s for *HS* with g = 2 and 5.5×10^{-5} s for *LS* with $g = 2.25$. The results reveal, besides a temperature independent T_2, a T_1 that depends exponentially on temperature ($T_1 \propto \exp[\Delta/kT]$) for both *LS* and *HS* states, pointing to an Orbach process at the common dominant relaxation mechanism. Our results for *HS* are in good agreement with previous work (1, 7, 8). However, as mentioned above, for the *LS* state the Raman process is expected to dominate since the closest excited state, necessary for the operation of Orbach processes, is too far above at ~1,000 cm^{-1}. In this sense it is noteworthy that the Orbach-like behavior of the *LS* state is only observed in samples where the *HS* state is also present as it has already been noticed by Allen et al.[2]

The similarity of the results obtained in mixed state mb and hb for the spin relaxation of Fe in the *LS* state suggests that the relaxation mechanism is associated with single heme complexes. This is expected because the magnetic interaction between Fe ions of different heme groups,

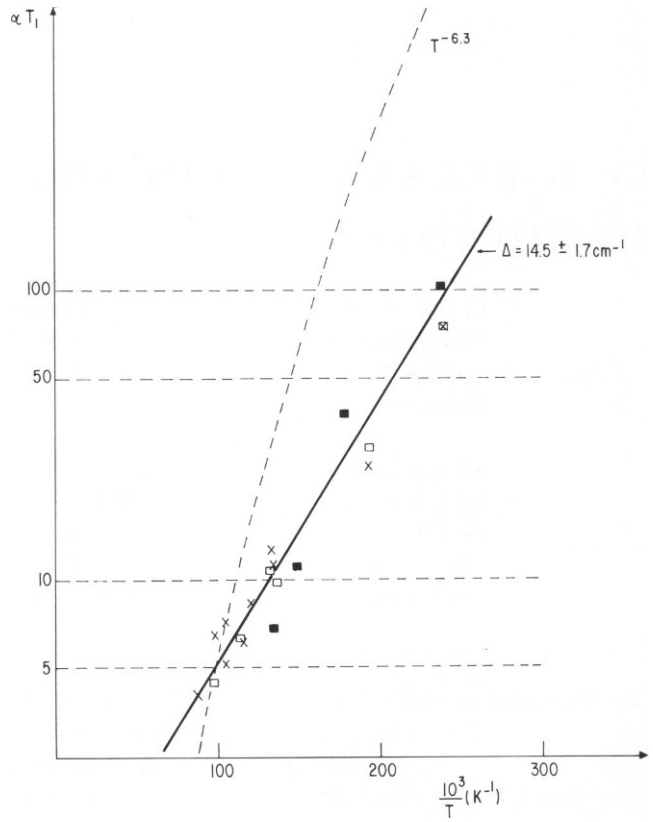

FIGURE 2 Temperature dependence of the relaxation time T_1 in met-hb. Data are normalized (see text). High spin $g = 6$ (■), $g = 2$ (□), low spin $g = 2.25$ (x).

TABLE I
VALUES OF T_2 IN NANOSECONDS FOR DIFFERENT HIGH SPIN AND LOW SPIN LINES OF MET-HB AND MET-MB FROM EQ. 2

	LS	*HS*	
Protein	$g = 2.25$	$g = 6$	$g = 1.99$
Met-hb	0.81 ± 0.1	0.74 ± 0.02	1.38 ± 0.15
Met-mb	0.90 ± 0.03	1.15 ± 0.15	1.36 ± 0.12

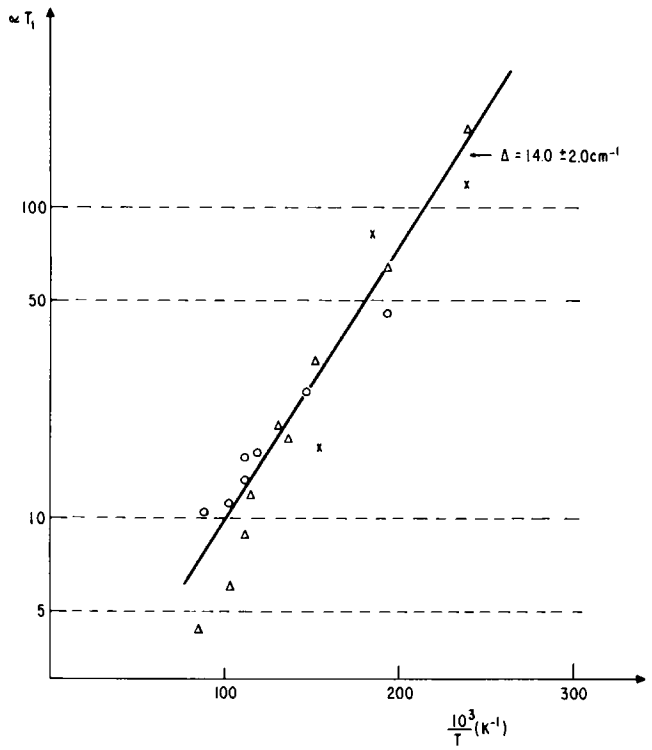

FIGURE 3 Temperature dependence of the relaxation time T_1 in met-mb. Data are normalized (see text). High spin $g = 6$ (x), $g = 2$ (Δ), low spin $g = 2.25$ (O).

which are ~30Å apart (12), is very small. An upper limit for the cross-relaxation of *LS* Fe towards the *HS* Fe ions in other heme groups can be estimated by the rate W_c of mutual spin-flip between neighboring Fe ions in *LS* and *HS* states. The order of magnitude of W_c induced by dipole interaction is given by (13)

$$W_c \simeq \left(\frac{5\mu_B^2}{\hbar r^3}\right)^2 R, \tag{4}$$

where r is a mean distance between heme groups in hb or mb crystals and

$$R = \frac{1}{\kappa}\int_{g_{LS}-\Delta g}^{g_{LS}+\Delta g} P_{HS}(g)\, P_{LS}(g)\, dg \tag{5}$$

is the overlapping factor of the *LS* and *HS* EPR spectra written in terms of the corresponding distribution functions of the g-factors, $P_{LS}(g)$ and $P_{HS}(g)$, respectively. Here $\kappa = \mu_B H/\hbar$, where H is the applied magnetic field. g_{LS} is the g-factor corresponding to the monitored ESR line and Δg its line width in terms of the g-factor. Assuming an ensemble of randomly oriented heme planes,[1] the calculation of $P_{LS}(g)$ and $P_{HS}(g)$ is straightforward (14, 15). Using the values $H = 3000G$, $r = 30$Å, $g_{LS} = 2.25$, $\Delta g = 0.02$ we obtain $\kappa R = 0.016$ and $W_c \lesssim 140 s^{-1}$. Since $W_c \ll 1/T_1$, cross-relaxation cannot account for the observed spin lattice relaxation of the *LS* state. In sum, the large distance between the hemes together with the small overlap between the spectral functions of the *LS* and *HS* states make cross-relaxation processes negligible. Thus, cross-relaxation between different paramagnetic centers, of the kind found in other biological systems (Calvo, R., W. F. Butter, D. R. Fredkin, M. Y. Okamura, and G. Feher. 1984. The electronic structure of FE^{2+} in reaction centers of *Rhodopseudomonas sphaeroids*. IV. Iron-assisted spin-lattice relaxation of the reduced primary quinone. Unpublished work.) is very improbable in this case.

An upper limit for the relaxation rate, W_i, of a *LS* state, induced by the time dependent magnetic dipole field of a neighboring *HS* state that relaxes through Orback processes is given by Eq. 4 with R replaced by $T_0/[1 + (\omega T_0)^2] \simeq 1/\omega^2 T_0)$, where $1/T_0$ is the Orbach relaxation rate. This yields $W_i \simeq 6 \times 10^{-8}/T_0$, which is also negligible compared to the observed relaxation rate ($\sim 10^{-2}/T_0$). Spin susceptibility measurements showing that the Curie law remains valid down to temperatures of the order of 20 mK (17) are consistent with these results. The magnetic interaction between an Fe ion and its neighboring nuclear spins is also too small to account for the observed relaxation rates (18, 19). We speculate, therefore, that in mixed state hemoproteins the dominant relaxation mechanism of *LS* Fe is induced by a local dynamic low spin-high spin interconversion rate (20). This interconversion rate must be less than the Larmor frequency ($\sim 10^{10}\ s^{-1}$) since discrete high and low spin spectra coexist. This idea seems also to be supported by Mössbauer measurements in mixed state met-mb where fluctuating hyperfine fields with characteristic times in the range 10^{-8}-10^{-9} s were observed (21).

In mixed state Fe compounds the crystal field is close to a critical value such that the LS and HS states have comparable free energies and can coexist (22). It is a situation of dynamical equilibrium where small variations of the crystal field (produced by vibrational modes) together with the spin-orbit interaction induce the interconversion of *LS* and *HS* states of Fe. Dynamic spin interconversion has been studied in ferric mb hydroxide (20) and in other six coordinated complexes of Fe (23, 24), where rates of the order of 10^7–$10^8\ s^{-1}$ were measured at about room temperature. Since the g-factors of the *LS* and *HS* states are quite different, the dynamic interconversion would produce a phase smearing of the precessional spins leading to a relaxation time T_2 of the order of the reciprocal interconversion rate. There are no independent measurements of interconversion rates of low temperatures to allow for a direct comparison with our surprisingly short values. However, the fact that a mixed state is present in the whole temperature range considered in this work, indicates that the activation energy for interconversion is small and therefore an almost temperature independent T_2 of the

[1]A more sophisticated calculation taking into account a possible correlation between the orientations of neighboring heme planes leads to essentially identical results.

order of those measured at room temperature may also be expected.

Although a LS-HS transition may always relax the transverse magnetization through the difference in g-factors, it will not in general relax the longitudinal magnetization. An interconversion transition with conservation of the longitudinal magnetization is already spin forbidden ($S = 1/2 \leftrightarrow S = 5/2$) and it must be assisted by the spin-orbit interaction. Thus, interconversion with longitudinal relaxation would involve terms of higher order in the spin-orbit interaction. More probably, the *LS* state relaxes to the lattice by interconversion to a *HS* state which subsequently suffers an Orbach relaxation before interconverting back to the *LS* state: this is suggested by the observation of similar activation energies Δ for the two spin states. Within this picture one would expect the relaxation rate of the *LS* state in mixed state hemoproteins to be given by

$$\frac{1}{T_1} = \frac{\alpha Q/T_0}{1+Q} + \frac{1/T_R}{1+Q}, \qquad (6)$$

where $Q = N_H/N_L$ is the ratio of the concentrations N_H and N_L of *HS* and *LS* states, respectively, in thermal equilibrium. $1/T_R$ is the Raman relaxation rate corresponding to pure *LS* states. In the temperature range considered in this work (between 4K and 14K) Q is a slowly varying function of T of the order of one, and T_1 is dominated by the exponential temperature dependence of T_0. However, since the magnitude of T_1 for *LS* states is 100 times larger than T_0 for *HS*, the efficiency of this process must be substantially smaller than one and we take this into account through the additional factor α. As the interconversion transitions involve small displacements of the iron in and out the plane of the heme[1], it could happen that the interconversion transition leaves the *HS* Fe in an excited vibrational state from which Orbach relaxation is less probable than from the ground vibrational state. In that case the longitudinal relaxation rate would not simply depend on the time the Fe spends in the *HS* state.

Although at present these considerations are purely speculative, they are susceptible of being tested experimentally and introduce aspects of the problem that necessarily will have to be taken into account in an elaborate model.

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REFERENCES

1. Scholes, C. P., R. A. Isaacson, and G. Feher. 1971. Determination of the zero-field splitting of Fe^{3+} in heme proteins from the temperature dependence of the spin-lattice relaxation rate. *Biochim. Biophys. Acta.* 244:206–210.
2. Allen, J. P., J. T. Colvin, D. G. Stinson, C. P. Flynn, and H. J. Stapleton. 1982. Protein conformation from electron spin relaxation data. *Biophys. J.* 38:299–310.
3. Colvin, J. T., G. C. Wagner, J. P. Allen, and H. J. Stapleton. 1984. Protein fractal dimensions and the density of low frequency vibrational modes. *Biophys. J.* 45(2, Pt. 2):130*a*. (Abstr.)
4. Helman, J. S., A. Coniglio, and C. Tsallis. 1984. Fractions and the fractal structure of proteins. *Phys. Rev. Lett.* 53:1195–1197.
5. Wajnberg, E., H. J. Kalinowski, and G. Bemski. 1985. High and low spin state mixture in methemoglobin and metmyoglobin. *An. Acad. Brasil. Cienc.* 57:1–5.
6. Wajnberg, E., H. J. Kalinowski, G. Bemski, and J. S. Helman. 1985. Spin relaxation of iron in mixed state hemoproteins. *Bull. Am. Phys. Soc.* 30:385.
7. Rupp, H., K. K. Rao, and R. Cammack. 1978. Electron spin relaxation of iron sulphur proteins studied by microwave power saturation. *Biochim. Biophys. Acta.* 537:255–269.
8. Castner, T. E. 1963. Raman spin-lattice relaxation of shallow donors in Silicon. *Phys. Rev.* 130:58–75.
9. Yim, M. B., L. C. Kuo, and M. W. Makinen. 1982. Determination of the zero-field splitting constants of high-spin metallproteins by a continuous-wave microwave saturation technique. *J. Magn. Res.* 46:247–256.
10. Blum, H., and T. Ohnishi. 1980. Electron spin relaxation of the electron paramagnetic resonance spectra of cytochrome *c*. *Biochim. Biophys. Acta.* 621:9–18.
11. Pattison, M. R., and Y. W. Kim. 1981. Distant-proton electron-nuclear double resonance in dilute paramagnetic systems. Case of metmyoglobin. *J. Magn. Res.* 43:193–206.
12. Dickerson, R. E., and I. Geis. 1983. Hemoglobin. The Benjamin Cummings Publishing Co., Inc. Menlo Park, CA.
13. Pake, G. E., 1962. Paramagnetic Resonance. W. S. Benjamin, Inc., NY. 129.
14. Poole, C. P., and H. A. Farach. 1972. The Theory of Magnetic Resonance. Wiley-Interscience Press, NY.
15. Weissbluth, M., 1967. The physics of hemoglobin. *Struct. Bonding.* 2:1–125.
16. Scholes, C. P., R. Janakiraman, H. Taylor, and T. E. King. 1984. Temperature dependence of the electron spin-lattice relaxation rate from pulsed EPR of Cu_A and heme a in cytochrome *c* oxidase. *Biophys. J.* 45:1027–1030.
17. Bemski, G., M. Novak, and O. G. Symko. 1983. Magnetization of Hemoglobin and Myoglobin below 1 K. *Phys. Lett.* 99A:62–64.
18. Hampton, D. A., and A. S. Brill. 1979. Crystalline state disorder and hyperfine component line widths in ferric hemoglobin chains. *Biophys. J.* 25:301–312.
19. Brill, A. S., and D. A. Hampton. 1979. Quantitative evaluation of contributions to electron paramagnetic resonance line widths in ferric hemoglobin single crystals. *Biophys. J.* 25:313–322.
20. Dose, E. V., M. F. Tweedle, L. J. Wilson, and N. Sutin. 1977. A direct measurement of dynamic spin-interconversion rates in the spin-equilibrium protein ferric myoglobin hydroxide. *J. Am. Chem. Soc.* 99:3886–3888.
21. Caughey, W. S., W. Y. Fujimoto, A. J. Bearden, and T. H. Moss. 1966. Mossbauer spectroscopy evidence for low-spin iron in dehydrated metmyoglobin. *Biochemistry.* 5:1255–1261.
22. Kotani, M. 1968. Paramagnetic properties and electronic structure of iron in heme proteins. Adv. Quantum Chem. 4:227–266.
23. Binstead, R. A., J. K. Beattie, E. V. Dose, M. F. Tweedle, and L. J. Wilson. 1978. Intersystem crossing observed by ultrasonic relaxation of the $^2t \rightleftharpoons {}^6A$ spin equilibrium of hexadentate Iron (III) complexes in solution. *J. Am. Chem. Soc.* 100:5609–5614.
24. Dose, E. V., M. A. Hoselton, N. Sutin, M. F. Tweedle, and L. J. Wilson. 1978. Dynamics of intersystem crossing processes in solution for six-coordinated d^5, d^6 and d^7 spin-equilibrium metal complexes of Iron (III), Iron (II) and Cobalt (II). *J. Am. Chem. Soc.* 100:1141–1147.