BRIEF COMMUNICATION

NMR RELAXATION OF PROTEIN AND WATER PROTONS IN METHEMOGLOBIN SOLUTIONS

MAURICE EISENSTADT, *Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461 U.S.A.*

ABSTRACT Hemoglobin (Hb) proton spins rapidly equilibrate among themselves after an initial excitation, and relax toward thermal equilibrium as a unit. In the diamagnetic form, spin diffusion to nearby methyl relaxation sinks can account for this. For metHb, four strong heme relaxation centers dominate, and spin diffusion must occur over long distances. A sizeable difference in protein T_1 is found between H_2O and D_2O solutions, much more than for diamagnetic Hb, consistent with internal H_2O acting as a spin carrier to the heme.

INTRODUCTION

In proton relaxation studies of diamagnetic proteins, it is striking that most spectral regions have the same longitudinal magnetization recovery rate after initial spin excitation (1). For the purposes of this study, then, we can treat all protein-proton spins as equivalent, or "well-mixed." Good mixing of solvent-water protons also occurs, because molecular diffusion is rapid, and the lifetime of a water molecule in the hydration region can be assumed to be short. Protein and water protons exchange magnetization at the protein-water interface via cross-relaxation processes (2, 3); direct transfer by proton exchange is negligible at our pH, and time scale ~0.1 s. Thus a simple two-phase exchange formalism can be used to describe relaxation, analogous to relaxation in systems with atomic exchange (4). Magnetization of each phase relaxes as the sum of two exponentials, whose slopes and amplitudes are functions of the number of protons and the intrinsic relaxation rates in each phase, and the exchange rates between them.

Our interest here is to investigate how efficiently the protein-proton spins mix among themselves, and to probe the mechanisms involved. Individual protons have varied interactions and molecular dynamics, and some, for example methyl protons, would be expected to have faster relaxation rates than others (1). However, overall equilibration is rapid, in part due to efficient spin coupling, but mainly because differences in local relaxation rates are small, and relaxation sinks such as methyl groups are widely distributed. This equilibration can be characterized as a diffusion process, similar to thermal or atomic diffusion, and theoretical spin-diffusion constants D_s have been derived for solid-state nuclear magnetic resonance (NMR) (5). Although D_s is always many orders of magnitude slower than solvent diffusion,

any reasonable estimate (see below) would probably be adequate to smooth out local T_1 variations.

The spin diffusion mechanism is more severely tested with methemoglobin, in which the heme irons are ferric, paramagnetic, and potent relaxation centers for the water spins (6). Most protein-protons are immobile and quite distant from the heme sites, and the value of D_s estimated below would leave them unaffected. However, we find that for methemoglobin, protein relaxation is enhanced, and much more so in H_2O than D_2O. The latter result suggests that there is enough mobile water in the protein interior to act as a spin carrier to the ferric relaxation sinks.

EXPERIMENTAL

Human erythrocytes were washed in isotonic saline-phosphate buffer at pH 5.7, hemolyzed by repeated freezing and thawing cycles, centrifuged (and sometimes filtered) to remove stroma or other suspended matter, and diluted to the desired protein concentration using the buffer mixture. Measurements with diamagnetic hemoglobin (Hb) were made soon after preparation, so that there was little spontaneous conversion to the met form, as indicated by the water T_2. MetHb was obtained by first incubating the red cells in 1% sodium nitrite, and then proceeding as above (7). Preparation of solutions in D_2O was similar, using reagents in 99.8% D_2O, freed of paramagnetic impurities by ion exchange. Red cells were incubated in D_2O for 24–48 h, which resulted in deuteration of most of the exchangeable protein-protons, (~20% of the total).

NMR relaxation measurements (T_1 and T_2) were made at 20 MHz, using a spectrometer described elsewhere (4). Longitudinal relaxation of a two-phase system with exchange is given in general by the sum of two exponentials (4):

$$\Delta M_P(t) = M_{P+}\exp(-\phi_{1+}t) + M_{P-}\exp(-\phi_{1-}t) \tag{1a}$$

$$\Delta M_A(t) = M_{A+}\exp(-\phi_{1+}t) + M_{A-}\exp(-\phi_{1-}t) \tag{1b}$$

$$2\phi_{1\pm} = (R_{1p} + k_s + R_{1a} + k_t) \pm [(R_{1p} + k_s - R_{1a} - k_t)^2 + 4k_sk_t]^{1/2}, \tag{2}$$

$$(\phi_{1+} - \phi_{1-})M_{P+} = (R_{1p} + k_s - \phi_{1-})\Delta M_{P0} - k_t\Delta M_{A0} \tag{3a}$$

$$(\phi_{1+} - \phi_{1-})M_{P-} = (\phi_{1+} - R_{1p} - k_s)\Delta M_{P0} + k_t\Delta M_{A0} \tag{3b}$$

$$(\phi_{1+} - \phi_{1-})M_{A+} = (R_{1a} + k_t - \phi_{1-})\Delta M_{A0} - k_s\Delta M_{P0} \tag{3c}$$

$$(\phi_{1+} - \phi_{1-})M_{A-} = (\phi_{1+} - R_{1a} - k_t)\Delta M_{A0} + k_s\Delta M_{P0} \tag{3d}$$

$$M_{P+} + M_{P-} = \Delta M_{P0} \equiv M_{P0} - M_P^0,\ M_{A+} + M_{A-} = \Delta M_{A0} \equiv M_{A0} - M_A^0. \tag{3e}$$

Magnetizations of the protein and water spins, M_P and M_A are expressed as deviations from equilibrium magnetizations M_P^0 and M_A^0, e.g., $\Delta M_P = M_P - M_P^0$. Initial values, after a preparative pulse sequence, are M_{P0} and M_{A0}. The fast and slow T_1 decay rates are ϕ_{1+} and ϕ_{1-}, respectively, and depend on the (single) intrinsic relaxation rates of the protein and water phases, R_{1p} and R_{1a}, and average spin-transfer rates from protein to water (k_s) and the reverse (k_t); the latter are related through detailed balancing, $k_sN_P = k_tN_A$, where N_P and N_A are the proton populations of the protein and solvent phases.

The central experimental problem is to extract the two decay rates, $\phi_{1\pm}$ from biphasic T_1 decays. (In addition, it is frequently possible to obtain enough amplitude information to evaluate R_{1p}, R_{1a}, and k_s separately.) For 2 mM protein concentrations in H_2O, $N_P/N_A = 0.12$, and in a conventional T_1 experiment, the fast component is barely discernible. Two techniques are used to obtain ϕ_{1+} (8): (*a*) The spin system is initially excited with a $90° - \tau - (-180°) - \tau - 90°$ *rf* pulse triplet, where τ is the time between pulses. Since T_2 of water is ~300 ms, and the protein T_2s are ~1 ms (data not shown), a τ of a

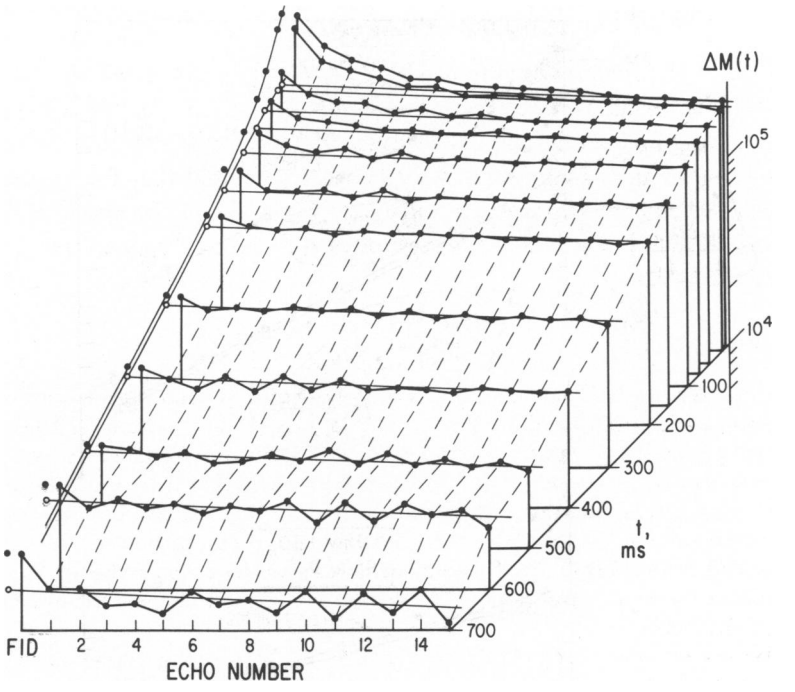

FIGURE 1 Representative magnetization recovery data for protein and water protons, monitored by a FID and spin-echo train, at various times t after an initial triplet excitation (τ = 5 ms); 2.4 mM metHb in H_2O, pH 6.0. Data points are connected as a visual aid.

few milliseconds gives $\Delta M_{P0} \simeq M_P$, $\Delta M_{A0} \simeq 0$. (*b*) Because of the long water T_2, later echos observed in monitoring the magnetization recovery with a spin-echo sequence (9, 10) are solely due to solvent spins. Extrapolation back to the start of the echo train gives $\Delta M_A(t)$; $\Delta M_P(t)$ follows by subtraction.

Similar techniques of selective excitation and detection were used in cross-relaxation studies by Edzes and Samulski (11). Fig. 1 shows raw magnetization recovery data of 2.4 mM metHb, pH 6.0 in H_2O, with triplet excitation and an echo monitor. The first signal of each pulse train is from a 50-μs gate beginning 50 μs after the start of the free induction decay (FID). Subsequent gates were 200 μs, centered on the echos, and spaced at 0.97 ms. The FID and extrapolated water signals are projected on the plane at the left, giving a graph similar to Fig. 2. In Fig. 1, the time evolution of each echo is shown by dashed lines, comprising a set of isoclines for the more distant echos. Early echos and the FID contain both protein and water signals; after subtracting ΔM_A, ΔM_P alone is shown (for a different sample) in Fig. 3, for the FID and first three echos. The fast exponential dominates (Eq. 1a), yielding ϕ_{1+}.

RESULTS

Fig. 2 shows relaxation data for Hb in H_2O, and 95% D_2O. The properties of this diamagnetic case will be treated in detail elsewhere, but some are needed below. Both inversion and triplet excitation were used. Approximate formulas for ϕ_{1+}, ϕ_{1-}, and M_{A+} are shown, valid for k_s, $k_t \ll R_{1p}$. The difference of ϕ_{1+} between H_2O and D_2O solutions is only $\sim 2\ s^{-1}$, attributed to differences in k_s rather than R_{1p}, and verified by the measured M_{A+}. Since k_s approaches zero for D_2O solvent, k_s in H_2O is $\sim 2\ s^{-1}$. For red cell suspensions, the effect of k_s can be shown directly, and is $\sim 4\ s^{-1}$ (Fig. 4, reference 2) for such highly concentrated Hb solutions.

Fig. 3 shows ΔM_P alone, for MetHb in H_2O and D_2O, as monitored by the FID and first

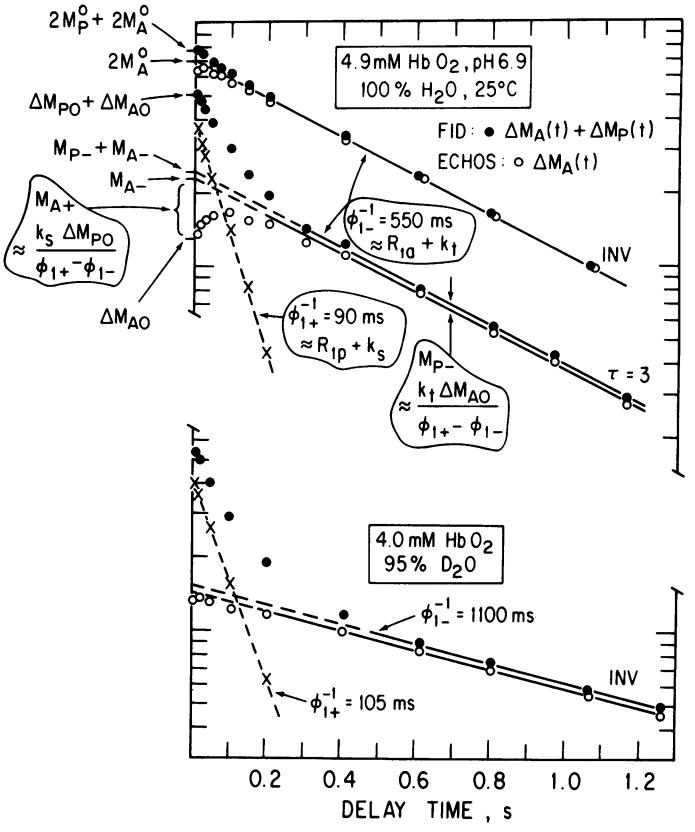

FIGURE 2 T_1 data for diamagnetic Hb in H_2O and D_2O after an initial inverting pulse or an *rf* triplet. Data are resolved into the components shown, as defined in the text.

three echo gates. Triplet excitation with τ = 1, 2.5, and 5 ms was used. For a given gate, comparison of slopes was made easier by normalizing all data to the same starting value. For τ = 1, only ~50% of the protein spins are excited; nevertheless, within 10 ms (H_2O) and 30 ms (D_2O), all monitors of one sample show the same single exponential behavior.

A further control is obtained if azide is bound to the sixth heme ligand site, blocking water access. This greatly decreases R_{1s} and ϕ_{1-}. Previous water relaxation studies (12) have shown azide-metHb to be indistinguishable from diamagnetic Hb. Although the magnetic moment of the azide complex is about half that of the aquo species, the electron relaxation time of the former is presumably much shorter. Azide binding greatly decreases ϕ_{1+} also (Fig. 3), but not to its diamagnetic limit; this contrast bears further investigation.

We did not study dependences on protein concentration in any detail. Although water relaxation is very sensitive to concentration (and pH), ϕ_{1+} was little affected until extreme concentrations.

DISCUSSION

First, consider diamagnetic relaxation of hemoglobin protons in D_2O solution. Typically $\phi_{1+}^{-1} \sim 100$ ms; this can only be due to spin interactions among the protein-protons themselves,

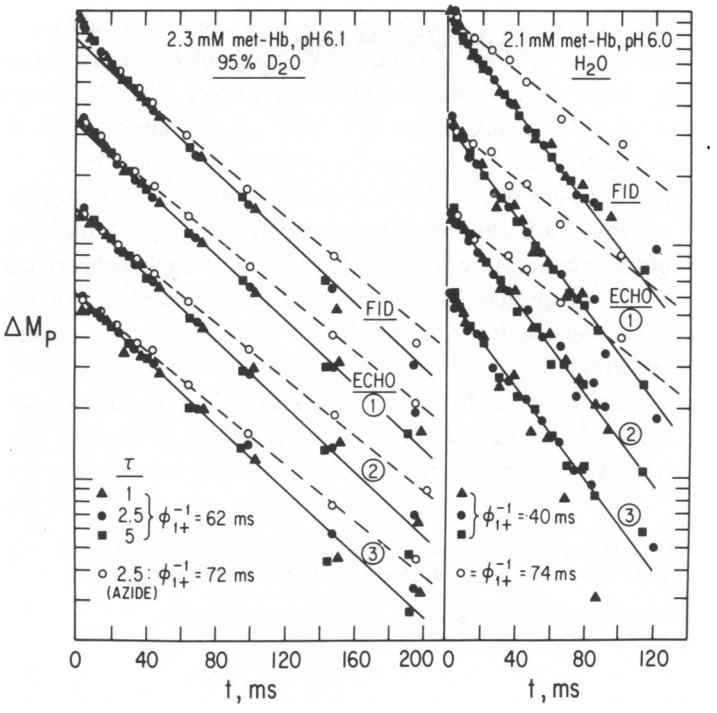

FIGURE 3 Magnetization recovery of protein-protons alone, after various triplets, for metHb in H_2O and D_2O, as monitored by the FID and first three echos. For the final data, the heme sites of both samples were saturated with azide.

mediated by spin diffusion, because the solvent deuteron spins couple very weakly to the protons. For paramagnetic metHb in D_2O, $\phi_1^{-1}{}_+$ decreases to ~60 ms; this difference provides a quantitative measure of spin transfer between protein-protons alone, a necessary starting point before evaluating any solvent contribution.

Spin diffusion in solutions is slower than in solids, since the tumbling of the solute molecules, or their internal motions give T_2 values much longer than the rigid lattice limit. We use the approximation $D_s = a^2/30\ T_2$ (5), where a is the distance between the coupled protons responsible for the process. Taking a = 2.5 Å, a typical distance between proton groups, and an average T_2 = 1.0 ms, $D_s = 2.1 \times 10^{-14}$ cm^2/s. The distance between ferric atoms ranges from 25 to 40 Å (13); taking 15 Å as the farthest distance of any proton from a relaxation center, and using the three dimensional diffusion formula (14) $t = l^2/6D_s$, the time for a spin to diffuse 15 Å is ~170 ms. Considering the many approximations, this value is consistent with the measured $R_{1\rho}$ in D_2O.

The contribution of spin diffusion for a solution of Hb or metHb in H_2O should be much the same as for D_2O. About 20% of the protein-protons are exchanged for deuterons in our D_2O samples, but this would make $R_{1\rho}$ somewhat faster than in a 100% protonated molecule, since for well-mixed protein spins, relaxation is proportional to the ratio of protein-protons to ferric atoms.

For metHb, ϕ_{1+} is 9 s^{-1} faster in H_2O than D_2O. Although solvent relaxation R_{1a} is

drastically affected by the paramagnetic hemes, ϕ_{1+} is almost independent of R_{1a} at 2 mM Hb concentration. Calculations with Eq. 2 using a wide range of R_{1a} verify that $\phi_{1+} \sim R_{1p} + k_s$. That is, for a solute-proton/solvent-proton population ratio of ~0.1, the more populous water phase behaves like an infinite sink. Since k_s is only ~2 s^{-1}, known from the diamagnetic experiment, most of the difference in ϕ_{1+} must arise from differences in R_{1p}.

We conclude that the faster R_{1p} in H_2O is due to solvent protons acting as spin carriers to the ferric relaxation sinks via pathways within the protein. It is probable that such pathways reach all portions of the protein, since from quantitative amplitude measurements we know we measure almost all of the protein spins. Few carriers are needed. An internal diffusion constant even approaching the free water value of 2×10^{-5} cm^2/s would enable a small number to have great effect.

It also follows that the lifetime of such carriers within the protein must be long compared with R_{1p}^{-1}. Any route to the heme site via the outside solvent would lead to immediate spin mixing with the water phase, and the carrier would only contribute to k_s.

The author is indebted to I. H. Scheinberg for his thorough reading of the manuscript.
This work was supported by National Institutes of Health grants HL 17571 and GM18298.

Received for publication 2 July 1980 and in revised form 18 November 1980.

REFERENCES

1. KALK, A., and H. J. C. BERENDSEN. 1976. Proton magnetic relaxation and spin diffusion in proteins. *J. Magn. Reson.* **24**:343–366.
2. EISENSTADT, M., and M. E. FABRY. 1978. NMR relaxation of the hemoglobin-water proton spin system in red blood cells. *J. Magn. Reson.* **29**:591–597.
3. KOENIG, S. H., R. G. BRYANT, K. HALLENGA, and G. S. JACOB. 1978. Magnetic cross-relaxation among protons in protein solutions. *Biochemistry.* **17**:4348–4358.
4. EISENSTADT, M. 1980. NMR hybrid relaxation methods of studying chemical, physical, and spin exchange. I. General theory and experimental methods. *J. Magn. Reson.* **38**:507–527.
5. ABRAGAM, A. 1961. The Principles of Nuclear Magnetism. ch. V and IX. Clarendon Press, Oxford, England.
6. LUMRY, R., H. MATSUMIYA, F. A. BOVEY, and A. KOWALSKY. 1961. The study of the structure and denaturation of heme-proteins by nuclear magnetic relaxation. *J. Phys. Chem.* **65**:837–843.
7. JAFFE', E. R. 1963. The reduction of methemoglobin in erythrocytes. *Blood.* **21**:561–572.
8. EISENSTADT, M. 1980. NMR hybrid relaxation methods of studying chemical, physical, and spin exchange, II. Suppression methods. *J. Magn. Reson.* **39**:263–274.
9. CARR, H. Y., and E. M. PURCELL. 1954. Effects of diffusion on free precession in nuclear magnetic resonance experiments. *Physical Rev.* **94**:630–638.
10. MEIBOOM, S., and D. GILL. 1958. Modified spin-echo method for measuring nuclear relaxation times. *Rev. Sci. Instrum.* **29**:688–691.
11. EDZES, H. T., and E. T. SAMULSKI. 1978. The measurement of cross-relaxation effects in the proton NMR spin-lattice relaxation of water in biological systems: hydrated collagen and muscle. *J. Magn. Reson.* **31**:207–229.
12. KOENIG, S. H., R. D. BROWN III, and T. R. LINDSTROM. 1980. Interactions of solvent with the heme region of methemoglobin and fluoro-methemoglobin. Symposium on interaction between iron and protein in oxygen and electron transport. Airlie House, Airlie, VA. In press.
13. PERUTZ, M. F. 1967. Structure and function of hemoglobin. *Harvey Lect.* **63**:213–261.
14. JOST, W. 1971. Diffusion. Basic phenomena and mechanism. *Biomembranes.* **3**:5–34.