PROTON LONGITUDINAL RELAXATION INVESTIGATION OF HISTIDYL RESIDUES IN HUMAN NORMAL ADULT HEMOGLOBIN

IRINA M. RUSSU AND CHIEN HO
Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213

ABSTRACT The longitudinal relaxation of the C2 protons of surface histidyl residues as well as other aromatic protons of human normal adult deoxyhemoglobin investigated at 360 MHz is discussed in terms of the theory proposed by Kalk and Berendsen for the proton longitudinal relaxation in proteins (Kalk, A., and H. J. C. Berendsen. 1976. *J. Magn. Reson.* 24:343–366). The role of the four paramagnetic iron atoms of deoxyhemoglobin as fast-relaxing sinks for the overall proton longitudinal relaxation is evaluated according to the model proposed by Bloembergen for the relaxation of nuclei in crystals containing paramagnetic centers (Bloembergen, N. 1949. *Physica.* 15:386–426). The results suggest that the effectiveness of the paramagnetic iron atoms of deoxyhemoglobin for the overall proton longitudinal relaxation is reduced as a result of slower spin diffusion and wide distribution of methyl groups within the hemoglobin molecule. Thus, deoxyhemoglobin provides a good model for investigating the influence of cross relaxation on proton longitudinal relaxation in proteins at the slow motion limit and in the presence of paramagnetic centers. For the C2 protons of surface histidyl residues, we show that the cross relaxation resulting from the interresidue dipolar interaction makes an important contribution to their longitudinal relaxation.

INTRODUCTION

The longitudinal relaxation rates (R_1) of protons in high-resolution NMR spectra have been used during the past decade to investigate molecular conformations and dynamics of a variety of proteins, such as ribonuclease (1–3), lyzosome (2, 4, 5), papain (3), bovine pancreatic trypsin inhibitor (5), as well as the binding of small ligands to macromolecules (1, 6, 7). In proteins, the longitudinal relaxation of protons was first characterized by single relaxation rates, R_1, which have been expressed as sums of the contributions from the dipolar interactions of the proton of interest with neighboring protons (1, 2, 4, 8). More recently, it has become clear that the proton longitudinal relaxation in protein solutions is significantly influenced by cross-relaxation effects. Cross relaxation between protein and solvent protons has been shown to influence greatly the longitudinal relaxation of the water protons (9–13). The influence of cross relaxation on the R_1 values of protein protons has been first thoroughly discussed by Kalk and Berendsen (3). They have shown that, at the slow-motion limit (e.g., proteins of molecular weight >10,000, at frequencies exceeding 200 MHz), the R_1 values of individual protons tend to become equal due to the mutual exchange of spin magnetization through cross relaxation. As a result of this effect, the R_1 values at the slow-motion limit cannot, in most cases, provide direct information on the specific motions and internuclear distances of individual protons within a protein. Kalk and Berendsen (3) have proposed that the longitudinal relaxation of individual protons in proteins is dominated by the relaxation of the rotating methyl groups. According to this model, in a large protein molecule at high frequencies, the R_1 values of all protons become the average of the longitudinal relaxation rates of methyl group protons multiplied by the ratio of the number of methyl protons to the total number of protons. Additional experimental evidence on the influence of cross relaxation on proton R_1 in proteins has been presented by Sykes et al. (5).

In the present work, we have studied the longitudinal relaxation of the C2 protons of surface histidyl (His) residues as well as other aromatic protons in the human adult hemoglobin (Hb A) molecule, in the deoxygenated state, at 360 MHz. Our interest in this study has been twofold . First, the deoxy Hb A molecule provides a good model for investigating the influence of cross relaxation on proton longitudinal relaxation in proteins at the slow-motion limit and in the presence of paramagnetic centers. Second, previous work from this laboratory has shown that the surface His residues of Hb can be used as excellent probes for the local conformations and environments in specific areas on the surface of the Hb molecule. According to the x-ray diffraction results in deoxy Hb A, there are 13 His residues on the surface of the molecule (14). Our

---

Address all inquires and reprint requests to Dr. Chien Ho, Department of Biological Sciences, 4400 Fifth Avenue, Carnegie-Mellon University, Pittsburgh, PA 15213.

BIOPHYS. J. © Biophysical Society · 0006–3495/82/08/203/08 $1.00
Volume 39 August 1982 203–210

previous investigation of the longitudinal relaxation of the surface His-C2 protons in deoxy Hb A and deoxy sickle hemoglobin (Hb S) has indicated that the R_1 of these protons contain valuable information on the differences in the surface conformation between Hb S and Hb A as well as on the molecular processes involved in the early stages of the polymerization of Hb S (15–17).

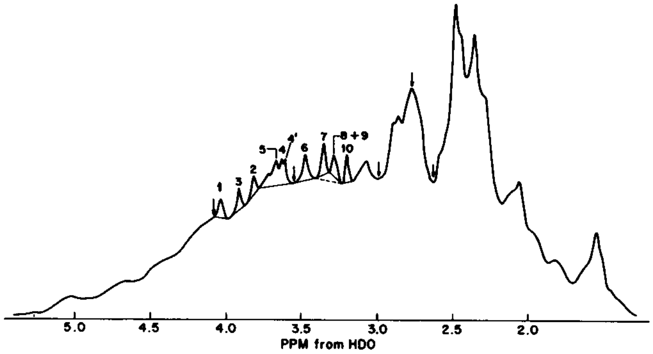

FIGURE 1 The procedure used for measuring the intensities of the sharp His proton resonances in a R_1 measurement. The arrows represent the spectral position used in the determination of the R_1 value of the aromatic envelope.

EXPERIMENTAL

Materials

Hb A was prepared by the standard procedure used in our laboratoy (18) from fresh blood samples obtained from the local Blood Bank (Pittsburgh, PA). Paramagnetic ion impurities were removed from the Hb solution using Chelex-100 (100–200 mesh, Bio-Rad Laboratories, Richmond, CA) by the procedure of Willard et al. (19). No differences in the UV-visible absorption spectra, the oxygen dissociation curves or the ^1H NMR spectra (i.e., hyperfine shifted, ring-current shifted, and aromatic proton resonances) were detected in the Hb samples before and after Chelex treatment. The deuterium oxide used throughout the present studies (99.8% in deuterium content, Bio-Rad) was glass distilled and any remaining metal ion impurities were extracted with 0.05% dithizone solution in carbon tetrachloride (20). The Hb samples in 0.1 M bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) buffer, in the deoxygenated state, were prepared as described previously (18, 21).

Methods

The longitudinal relaxation rates of the aromatic proton resonances were measured at 360 MHz using a spectrometer (Bruker Instruments, Inc., model WH-360/180) spectrometer located at the University of Pennsylvania. The spectrometer was locked on the solvent ^2H signal. The quadrature detection mode was used. Each spectrum was the result of averaging 200 scans. The measurement of the R_1 values was carried out using an inversion-recovery method with the following pulse sequence: $(180° - t - 90° - t_w)_n$. The waiting time, t_w, between two successive pulse sequences was 4.7 s; ten values for the time interval t in the range from 0.02 to 3.5 s were used. The length of the 90° pulse was determined using the proton resonance of the residual water in a Hb sample and was found to be 8.3 μs.

The ^1H NMR spectrum of Hb over the region 1.5 to 5.0 ppm downfield from the proton resonance of HDO contains two components as shown in Fig. 1: (*a*) the broad aromatic resonances whose envelope can be treated, in the first approximation, as a single relaxing component; and (*b*) the sharp resonances, such as the surface His-C2 proton resonances labeled from 1 to 10 in Fig. 1. We have found that the R_1 values for each of these components cannot be determined accurately by using the total intensity at a given frequency, for the following two reasons: (*a*) the relative contribution of the sharp component to the total intensity is much less than that of the broad component (Fig. 1); and (*b*) the ratio between the R_1 values of the broad and sharp components is very close to unity (see Results). Consequently, in the present work, the R_1 values were calculated by measuring the intensities of the sharp and broad components, separately. The procedure used is illustrated in Fig. 1. For the broad component, the intensity was measured in the five spectral positions labeled by arrows in Fig. 1. For the sharp resonances, the intensities were measured using the base line of the broad aromatic resonance envelope (Fig. 1). In this procedure, the base line is to a certain extent arbitrarily defined (see, for example, resonances 7 and 8 + 9 in Fig. 1) and we have found that, depending on the base line chosen, the R_1 values of the sharp His resonances are distributed within 10 to 15% from each other. Furthermore, given this limitation in the intensity measurements, the present experimental data for the His resonances do not allow a detailed analysis of the relaxation curves in terms of sums of two exponential functions which characterize the longitudinal relaxation of these resonances. Consequently, the R_1 values of the His-C2 protons were obtained by fitting the intensity of the corresponding resonance, I, as a function of time to the single exponential equation: $I = I_0 (1 - 2 \cdot c^{-R_1 \cdot t})$, where I_0 is the equilibrium intensity.

RESULTS

We have measured the longitudinal relaxation rates of the aromatic proton resonances of deoxy Hb A solutions in 0.1 M Bis-Tris buffer in D_2O at pH 6.80. The measurements have been carried out as a function of the Hb concentration (from 10 to 17% [wt/vol]) and of the temperature (from 17° to 37°C). A representative longitudinal relaxation curve for the surface His-C2 protons is shown in Fig. 2. The relaxation curve for the broad aromatic resonances is presented in Fig. 3. Table I shows several sets of R_1 values for the surface His-C2 protons. The corresponding relaxation rate for the broad aromatic resonances under these experimental conditions is 1.2 ± 0.1 s^{-1}.

TABLE I
LONGITUDINAL RELAXATION RATES OF THE HIS-C2 PROTONS IN 13.5% DEOXY HB A

Resonance no.	R_1 (s^{-1})		
	17°C	*25°C*	*37°C*
1	1.2	1.2	0.9
2	0.8	0.8	0.7
3 (β146 His-C2)	0.8	0.7	0.6
4	0.9	0.8	0.6
4′	0.9	0.8	0.6
5	0.9	0.9	0.8
6	0.8	0.7	0.6
7	1.3	1.0	0.9
8 + 9 (β143 His-C2)	0.9	0.8	0.7
10 (β2 His-C2)	1.4	1.6	1.3

Solutions in 0.1 M Bis-Tris buffer at pH 6.80 as a function of temperature. The accuracy of the R_1 measurements is ±0.1 s^{-1}.

DISCUSSION

In the present work, we have studied those protons of Hb that give rise to resonances in the aromatic proton resonance region of the ^{1}H NMR spectrum (1.5 to 5.0 ppm downfield from HDO as shown in Fig. 1). The sharp aromatic resonances labeled 1–10 in Fig. 1, which are of special interest to this work, originate from the C2 protons of His residues situated on the surface of the Hb molecule (15, 17). The broad resonances present in the aromatic proton resonance region originate from "bulk" protons of Hb, such as protons of the aromatic amino acid residues situated in the interior of the Hb molecule (17) and protons of the NH groups not exchanged with solvent deuterons (17, 22).

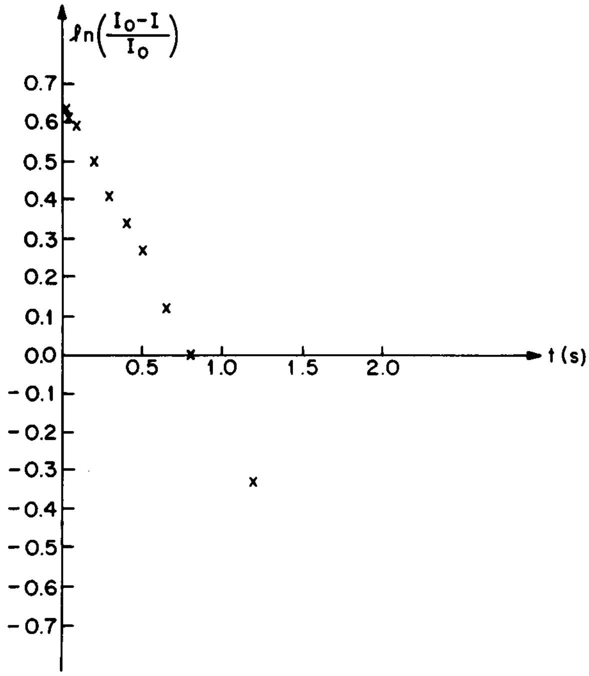

Figure 2 Longitudinal relaxation for resonance 5 in 13.5% deoxy Hb A in 0.1 M Bis-Tris buffer at pH 6.80 and at 25°C.

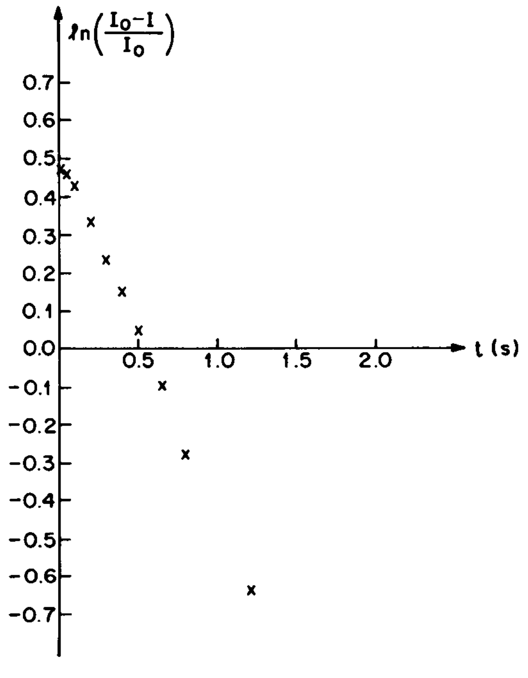

Figure 3 Longitudinal relaxation of the bulk protons in 10% deoxy Hb A in 0.1 M Bis-Tris buffer at pH 6.80 and at 25°C.

In the following, we assume that the physical mechanisms responsible for the longitudinal relaxation of these aromatic protons of Hb are: (*a*) the intramolecular dipolar interaction between the protons observed and adjacent protons of Hb; and (*b*) the dipolar interaction between any given proton of Hb and the unpaired electrons of the paramagnetic iron atoms. The dipolar interaction between any Hb proton and other nuclei (of Hb or from solvent), such as ^{2}H, ^{13}C, or ^{14}N, is neglected since the gyromagnetic ratio of these nuclei is small relative to that of ^{1}H.

Longitudinal Relaxation of the "Bulk" Protons in Deoxy Hb A

By monitoring the envelope of the broad aromatic resonances in the ^{1}H NMR spectrum of deoxy Hb A, we have found that for all five spectral positions analyzed (see Fig. 1), the longitudinal relaxation curves are the same and they correspond to a relaxation rate of 1.2 ± 0.1 s^{-1}. As pointed out by Kalk and Berendsen (3), this finding clearly suggests that the longitudinal relaxation of the "bulk" protons of deoxy Hb A is influenced primarily by the averaging effects of cross relaxation.

The cross-relaxation process plays an important role in the longitudinal relaxation of the bulk aromatic protons of Hb A because the correlation time for the overall rotation of the Hb molecule τ_c (4.3×10^{-8}s in a 10% deoxy Hb A solution in 0.1 M Bis-Tris buffer in D_2O at 25°C [23]) corresponds to the slow-motion limit at the proton observation frequency of 360 MHz, i.e., $(\omega_H \cdot \tau_C)^2 \simeq 10^4 >> 1$. At this limit, the main effect of the dipolar interaction among protons is to cause simultaneous transitions of antiparallel spins such that a mutual exchange of magnetization occurs, at a rate much larger than the corresponding spin-lattice relaxation rate. Under these conditions, the energy of the spin system is dissipated to the lattice through the fast-relaxing sinks present in the molecule. In deoxy Hb A, two types of fast-relaxing sinks are expected to be most important for the overall proton longitudinal relaxation: (*a*) the protons of the methyl groups (3) and (*b*) the unpaired electrons of the iron atoms. Kalk and Berendsen (3) have shown that, due to the fast internal motion of the methyl groups, the spin-lattice relaxation rate of their protons is greatly enhanced as compared with that of the protons held fixed relative to the protein molecule.

Thus, in diamagnetic proteins, the proton longitudinal relaxation becomes limited by the spin-lattice relaxation rates of the protons of methyl groups.

The influence of the unpaired electrons of the iron atoms on the proton longitudinal relaxation in deoxy Hb A can be evaluated following the model proposed by Bloembergen (24) for the spin relaxation of nuclei in crystals containing paramagnetic centers. The following two parameters are important in this model: (*a*) the proton spin-lattice relaxation rates resulting from the interaction with the unpaired electrons of the paramagnetic atom and (*b*) the spin diffusion constant. For a dipolar coupling, the paramagnetic contribution to the spin-lattice relaxation rate of the ith proton R_i^* is given as (25)

$$R_i^* = \frac{2}{15} \cdot \frac{\gamma_H^2 \cdot g^2 \cdot \beta^2}{r_i^6} \cdot S \cdot (S+1) \cdot \left(\frac{3\tau_p}{1 + \omega_H^2 \tau_p^2} + \frac{7\tau_p}{1 + \omega_S^2 \tau_p^2} \right) \quad (1)$$

where γ_H is the proton gyromagnetic ratio, ω_S is the electron Larmor frequency, S is the total electronic spin of an iron atom ($S = 2$ for deoxy Hb A), r_i is the distance between the ith proton and the iron atom, β is the Bohr magneton, and g is the electronic Landé factor (taken here as that of a free electron). In the case of deoxy Hb A, the corresponding correlation time τ_p is given by:

$$1/\tau_p = 1/\tau_c + 1/\tau_s \simeq 1/\tau_s,$$

where τ_c is the correlation time for the overall rotation of the Hb molecule (taken as 4.3×10^{-8} s [23]) and τ_s is the longitudinal relaxation time of the electronic spin (taken as 6.1×10^{-12} s [23]). Because $(\omega_S \cdot \tau_p)^2 >> 1$ and $(\omega_H \cdot \tau_p)^2 << 1$, for the present work, Eq. 1 can be simplified in terms of Bloembergen's model to:

$$R_i^* = C \cdot r_i^{-6} \quad (2)$$

where $C = 2/5 \cdot \gamma_H^2 \cdot g^2 \cdot \beta^2 \cdot S \cdot (S+1) \cdot \tau_p = 3.6 \times 10^{-42}\ \text{cm}^6 \cdot \text{s}^{-1}$.

The proton spin diffusion constant, D, is given by $D = W_0 \cdot a^2$, where W_0 is the probability of simultaneous transitions of antiparallel spins and a is the distance between two nearest protons (26). Since for any two Hb protons, i and j, $(\omega_{H,i} - \omega_{H,j})^2 \cdot \tau_c^2 << 1$ (or $[\omega_{H,i} - \omega_{H,j})] << 2 \times 10^7$ Hz) and taking a as 2.5 Å, the cross relaxation rate W_0 in Hb A is $\sim 10\ \text{s}^{-1}$ according to Solomon's equations for the relaxation in a system of two spins (27). Thus, the spin-diffusion constant, D, in Hb A is on the order of $6 \times 10^{-15}\ \text{cm}^2 \cdot \text{s}^{-1}$.

A detailed analysis of the spatial distribution of the magnetization and its flow to the paramagnetic sinks using Bloembergen's model (24) has been given by de Gennes (28) and Blumberg (29). Blumberg (29) has shown that for very short times following the disturbance of the spin system [i.e., $\sqrt{6Dt} << (Ct)^{1/6}$ or $t << 6^{-3/2} \cdot C^{1/2} \cdot D^{-3/2}$, when there is no gradient of the magnetization and, thus, diffusion cannot be important], the longitudinal relaxation of protons is mainly determined by their interaction with the nearest paramagnetic atom (Eq. 2). As a result, for each value of time t in this range, the nuclear magnetization is distributed such that within a sphere of radius $r = (Ct)^{1/6}$ around each paramagnetic atom, most of the protons have returned to equilibrium. Applying these conclusions to the case of deoxy Hb A, one can see that for the shortest time interval investigated here, ($t = 0.02$ s), the Hb protons situated within a range of ~6 Å from each iron atom have already reached thermal equilibrium. It is very likely that these protons have not been observed in the present experiments as their resonances are shifted by the paramagnetic effect outside the aromatic region of the ^{1}H NMR spectra (30). However, these protons clearly contribute through spin diffusion to the longitudinal relaxation of the rest of the Hb protons occurring at later times. De Gennes (28) has shown that for $t >> 0.5\ C^{1/2}\ D^{-3/2}$, the solution for the local magnetization in Bloembergen's model can be approximated as follows: within a sphere of radius $\rho = 0.68\ C^{1/4}\ D^{-1/4}$ (called "pseudopotential radius") around each paramagnetic center, all protons can be assumed to be at equilibrium; for the protons outside this sphere the decay of the longitudinal magnetization is then described, in the absence of the RF field, by the spin diffusion directed towards the paramagnetic centers. De Gennes' solution should be applicable to the Hb spin system only as a first approximation as the distances between the iron atoms in deoxy Hb A, ranging from 25 to 42 Å (14) do not greatly exceed the pseudopotential radius ($\rho \sim 10$Å for deoxy Hb A). Given the position of their resonances in the ^{1}H NMR spectra, most of the bulk aromatic protons of Hb A investigated here are expected to be situated outside the pseudopotential radius around each paramagnetic iron atom. Therefore, de Gennes' solution suggests that, if the longitudinal relaxation of the bulk protons were dominated by the spin diffusion towards the iron atoms, the R_1 values of the bulk protons should be less than those observed experimentally and their relaxation should occur mainly for times longer than $0.5\ C^{1/2}\ D^{-3/2} = 1.8$ s. This observation thus indicates that the longitudinal relaxation of the bulk aromatic protons in deoxy Hb A is also influenced to an appreciable extent by the spin-lattice relaxation of the methyl group protons. The spin-lattice relaxation rate of a methyl group proton in the Hb A molecule can be estimated at $\sim 4\ \text{s}^{-1}$, assuming a correlation time for the internal rotation of 5×10^{-10} s (5) and using Woessner's equations for relaxation in internally rotating groups (31). This value therefore shows that the spin diffusion to nearby methyl groups could influence the longitudinal relaxation of the bulk aromatic protons of deoxy Hb A to an extent comparable to the long-range spin diffusion to protons situated within the pseudopotential radius around each paramagnetic iron atom. Thus, it appears that, in deoxy Hb A, the paramagnetic contribution to the longitudinal relaxation of protons situated further away from the iron atoms is diminished compared to similar cases observed in solid-state NMR for the following reasons: (*a*) the spin diffusion in the Hb molecule, in solution, is slower than that in solids, and (*b*) the methyl groups are widely distributed within the Hb molecule.

The influence of the paramagnetic iron atoms on the longitudinal relaxation of protons in Hb A has also been recently investigated by Eisenstadt (13). Consistent with the present observations, Eisenstadt (13) has found that, at 20 MHz, the longitudinal relaxation rate of the proton resonance of Hb in D_2O is enhanced by ~70% in methemoglobin as compared with oxyhemoglobin. This result indicates that, even at this lower frequency, the proton longitudinal relaxation in Hb is greatly influenced by spin diffusion and the bulk of the protons have the same R_1 value. Furthermore, Eisenstadt (13) has observed that, in the met form, the R_1 value of the proton resonance of Hb is increased by ~50% in H_2O as compared with D_2O. Based on this result, he proposed as an additional way for the diffusion of the magnetization to occur that, in met Hb A in H_2O, the solvent protons act as spin carriers to the ferric relaxation sinks via pathways within the protein molecule.

TABLE II
PARAMAGENTIC DIPOLAR CONTRIBUTION TO THE LONGITUDINAL RELAXATION RATES OF THE SURFACE HIS-C2 PROTONS IN DEOXY HB A

His residue	Distances of the C2 proton to the iron atoms (Å)				R_1^*
	α_1 chain	α_2 chain	β_1 chain	β_2 chain	(s^{-1})
α20	23.4	47.2	39.7	44.4	0.02
α45	10.2	41.2	41.6	19.9	3.31
α50	20.1	46.1	36.5	33.4	0.06
α72	19.2	38.3	44.4	42.4	0.07
α89	14.6	32.3	43.6	24.2	0.38
α112	21.1	42.3	27.4	39.3	0.05
β2	29.4	40.7	27.9	24.4	0.03
β77	42.0	39.8	17.4	40.9	0.13
β97	42.0	17.8	13.4	40.6	0.75
β116	28.3	35.9	23.5	44.2	0.03
β117	31.9	37.2	20.7	45.1	0.05
β143	37.4	29.6	17.8	29.2	0.13
β146	42.9	40.3	17.4	35.9	0.14

Longitudinal Relaxation of the Surface His-C2 Protons in the Deoxy Hb A Molecule

The R_1 values of the eleven His-C2 proton resonances present in the aromatic proton resonance region of the ^1H NMR spectra of deoxy Hb A span from 0.6 to 1.6 s^{-1} (Table I). This range of values is appreciably narrower than what one would expect for the diverse local environments of the surface His residues in Hb that have been suggested by other experimental results, such as their ^1H NMR titration (15, 32). Furthermore, this range of R_1 values appears to be centered around the value for the longitudinal relaxation rate of the bulk protons (1.2 ± 0.1 s^{-1}).

The contribution to the R_1 values of the His-C2 protons from the direct dipolar interaction with the unpaired electrons of the iron atoms is shown in Table II. The first columns of Table II contain the distances between the C2 protons of the surface His residues of the $\alpha_1\beta_1$ dimer to the four iron atoms of Hb. A symmetrical relationship holds for the His residues of the $\alpha_2\beta_2$ dimer. These distances are calculated based on the x-ray coordinates of the iron atoms and those of the C2 carbons of surface His residues of deoxy Hb A (M. F. Perutz, personal communication). The coordinates of the His-C2 protons are obtained from the x-ray coordinates of the His-C2 carbons using the GENATM program written by Dr. R. Shiono of the University of Pittsburgh. The total contribution of the four iron atoms to the R_1 values of surface His-C2 protons is calculated based on Eq. 2 (last column of Table II). The results show that the R_1 values of two surface His residues, α45 His and β97 His, should be greatly affected by the paramagnetic dipolar interaction. However, it has been shown that, due to the ring-current effect of the porphyrin structure in the α and β chain, the C2 proton resonances of α45 His and β97 His are very likely shifted from the spectral region 1.5 to 5 ppm from HDO (17). For the rest of the eleven surface His residues (with the exception of α89 His), the paramagnetic contribution to the R_1 values of their C2 protons is negligible. Thus, the longitudinal relaxation of these His-C2 protons should result from their dipolar interactions with neighboring protons. However, in contrast with the aromatic protons situated in the interior of the Hb molecule, a surface His residue has the freedom of internal motions and the correlation times for these internal motions can be much shorter than that for the overall rotation of the Hb molecule. Thus, as for the protons of the rotating methyl groups (3), the internal motions of the surface His residues could be expected to enhance the spin-lattice relaxation rates of the His-C2 protons. To gain an insight into the specific contributions from the spin-lattice energy exchange and the cross relaxation to the R_1 values observed experimentally, we have analyzed the longitudinal relaxation of a surface His-C2 proton for both intraresidue and interresidue dipolar interactions for the following two cases: (*a*) a His residue held fixed relative to the Hb molecule, and (*b*) a His residue rotating internally around its Cβ—C5 bond. The results of this analysis are presented in Fig. 4. The effects of the dipolar interaction between the surface His-C2 proton and the solvent protons are not included here because, in all the present experiments, the solvent used was fully deuterated. For the case of a His residue held fixed relative to the Hb molecule, the spin-lattice and the cross relaxation rates, R and R_t, respectively, were calculated based on Solomon's equations for relaxation in a two-spin system (27), by using the following distances between the His protons: r(C2H—C4H) = 4.118 Å, r(C2H—CβH) = 4.632 Å, r(C2H—Cβ'H) = 4.372 Å, r(C4H—CβH) = 2.577 Å, and r(C4H—Cβ'H) = 3.230 Å (S. Takahashi, personal communication). For the case of a His residue rotating internally around its C5—Cβ bond, we have used the equations derived by Woessner (31); the angles Δ between the dipolar vectors and the axis of rotation were estimated according to the available structure of a His amino acid (S. Takahashi, personal communication) as follows: Δ(C2H—C4H) $\simeq$ 54°, Δ(C2H—CβH) $\simeq$ 16° and Δ(C4H—CβH) $\simeq$ 25°. The longitudinal relaxation curves for the case of the intraresidue dipolar interaction between the His-C2, the His-C4 and the two His-Cβ protons were obtained by solving the relaxation equations given in (3) for the longitudinal magnetization of the His-C2 proton. The longitudinal relaxation rate for the two Cβ protons was taken as being equal to that of the bulk protons (1.2 s^{-1}) because for these protons the distances to the Hb multispin system are shorter and, thus, the averaging effects of cross relaxation should be dominant.

As clearly shown in Fig. 4, the intraresidue dipolar interaction within a His residue, both held fixed relative to the Hb molecule or internally rotating, does not represent a process strong enough to account for the R_1 values observed experimentally. The presence of the internal motion has the effect of enhancing the spin-lattice relaxation rates, R, of the His protons as compared with the case of a fixed His residue as follows: R(C2H) from 7×10^{-4} s^{-1} to 2.2×10^{-2} s^{-1}, and R(C4H) from 7×10^{-3} s^{-1} to 0.1 s. This increase of about two orders of magnitude in the R values of the His-C2 and the His-C4 protons originates from the fact that the angle Δ between the dipolar vector C2H—C4H and the axis of rotation (~54°) is very close to the "magic angle" for which the contribution of the slow overall rotation of the Hb molecule to the spectral density function is greatly dimished (see reference 31). However, the internal rotation of the His residue has also the effect of largely decreasing the cross relaxation rate between the His-C2 and the His-C4 protons from 0.5 s^{-1} when the His residue is held fixed relative to the Hb molecule to $\sim -2.5 \times 10^{-3}$ s^{-1} in the presence of internal rotation. Due to this significant decrease in the cross relaxation rate, the R_1 value resulting from the intraresidue dipolar interaction remains even in the presence of internal motion very close to that predicted for a fixed His residue.

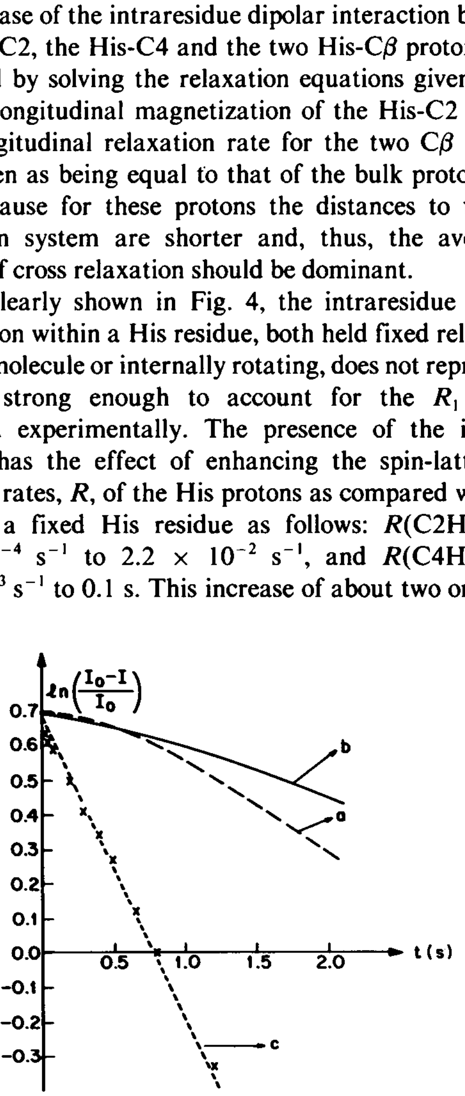

FIGURE 4 Longitudinal relaxation of a His-C2 proton for: (*a*) intraresidue dipolar interaction and a His residue held fixed relative to the Hb molecule; (*b*) intraresidue dipolar interaction and a His residue rotating around the C5-Cβ bond with a correlation time for internal rotation of 4.4×10^{-10} s; and (*c*) interresidue dipolar interaction for a His residue held fixed relative to the Hb molecule and in van der Waal's contact with three adjacent protons. The experimental points for resonance labled 5 (x) shown in Fig. 2 are also presented for comparison.

The interresidue dipolar interaction is considered here for the case in which the His residue is held fixed relative to the Hb molecule, assuming that the His-C2 proton interacts with n adjacent protons of neighboring amino acid residues. Such a conformation of a surface His residue is likely to exist in the Hb molecule since, according to the atomic model of deoxy Hb A, many of the surface His residues are situated in the vicinity of negatively charged amino acid residues; the resulting electrostatic interaction could be expected to bring the His-C2 proton closer to other protons of Hb. For the neighboring protons interacting with the His-C2 proton, we could assume a longitudinal relaxation rate close to that averaged over the Hb spin system by strong cross relaxation effects. Then, following Kalk and Berendsen (3), the R_1 value of the His-C2 proton tends to the average $[n/(n+1)] \cdot 1.2$ s^{-1}, where n is the number of adjacent protons of neighboring amino acid residues. Therefore, at the limit of strong cross relaxation, the interresidue dipolar interaction can predict R_1 values of the His-C2 protons in a range comparable to that observed experimentally, from 0.6 to 1.2 s^{-1}. As an example, Fig. 4 shows the longitudinal relaxation rate for resonance labeled 5, calculated under the assumption that the corresponding His-C2 proton is situated in van der Waal's contact with three adjacent protons. R_1 values of the His-C2 protons >1.2 s^{-1} could result, at the limit of strong cross relaxation, from the interresidue dipolar interaction between a His-C2 proton and a fast-relaxing sink, such as a methyl group.

Undoubtedly, the coupling of the His-C2 proton with protons of neighboring amino acid residues is modulated in time not only by the overall rotation of the Hb molecule but also by a large diversity of internal motions. The effects of such internal motion (e.g., rotations around the C5—Cβ and the Cβ—Cα bonds, small jumps in the magnitude of the internuclear separation vectors, etc.) on the interresidue dipolar interactions have been discussed in detail elsewhere (17). In general, they appear to be similar to the effects presented here for the intraresidue dipolar interactions and, in these cases also, the interresidue cross relaxation makes the dominant contribution to the R_1 values of the surface His-C2 protons.

The observations made here on the R_1 values of the surface His-C2 protons in deoxy Hb A can be exemplified by the longitudinal relaxation observed experimentally for β2 His-C2 proton (resonance labeled 10) and for β146 His-C2 proton (resonance labeled 3). The β2 residue is situated in the Hb A molecule next to the β1 Val and β3 Leu residues. Hence, the large R_1 value of the β2 His-C2 proton (Table I) could be explained as resulting from the interresidue cross relaxation between the His-C2 proton and the protons of the four methyl groups of β1 Val and β3 Leu residues (possibly also the methylene groups). According to the x-ray crystal structure of the deoxy Hb A molecule (M. F. Perutz, personal communication and reference 14), the β146 His-C2 proton is situated within ~4.5 Å of the following protons: two Cβ protons of β94 Asp, two Cβ protons of β93 Cys, and two Cβ protons of β90 Glu. Such methylene-type protons have been proposed to function as fairly fast-relaxing sinks for the longitudinal relaxation of protons in proteins (3). In the case of β146 His residue of Hb, because the inter-nuclear distances involved are quite large, the cross relaxation rates between the β146 His-C2 proton and the neighboring six methylene-type protons are expected to decrease. As a result, the R_1 value of β146 His-C2 proton (Table I) should be lowered compared with that averaged over the Hb multispin system.

In our work, we have observed a slight decrease in the R_1 values of several surface His-C2 protons upon raising the temperature (Table I). This could be due to a decrease in the spin-lattice relaxation rates of the fast-relaxing sinks of the molecule, such as the methyl group protons upon raising the temperature (if the correlation time for the internal motion of the methyl groups corresponds to the fast motion limit, i.e., $<4.4 \times 10^{-10}$ s). This effect may be enhanced by a decrease in the correlation times for the overall rotation of the Hb molecule and for the internal motions of the surface His residues, upon raising the temperature. No significant variation in the R_1 values of the large majority of surface His-C2 protons has been observed experimentally when the Hb concentration was raised from 10 to 17% (results not shown). This finding indicates that the intra-molecular dipolar interactions in a deoxy Hb A solution of Hb concentrations investigated here do not make a significant contribution to the longitudinal relaxation of the His-C2 protons. In contrast, in deoxy Hb S, the R_1 values of some of the His-C2 protons are strongly dependent on the Hb S concentration (15–17).

In conclusion, the analysis made here indicates that, although the surface His residues are commonly viewed as being magnetically isolated and involved in internal motions, one of the processes that can account for the longitudinal relaxation rates of their C2 protons in deoxy Hb A is the cross relaxation between the His-C2 protons and the protons belonging to neighboring amino acid residues. Thus, the present results emphasize once more the important role played by the cross relaxation process in the nuclear magnetic longitudinal relaxation of protons in proteins.

We wish to thank Dr. Mario Englesberg, Dr. Martin P. N. Gent, and Dr. Irving J. Lowe for helpful discussions. We are grateful to Dr. Max F. Perutz for providing us the x-ray coordinates of human deoxyhemoglobin and to Dr. Ryonosuke Shiono for providing us the GENATM program for generating the coordinates of the His-C2 protons. We also thank Dr. George G. McDonald for instructing and assisting us in the operation of the Bruker WH-360/180 NMR spectrometer.

This work is supported by research grants from the National Science Foundation (PCM78-25818) and the National Institutes of Health (HL-24525). The Bruker WH360/180 NMR spectrometer in the Middle Atlantic NMR Facility, University of Pennsylvania, Philadelphia, Pennsylvania, is supported by a grant from the National Institutes of Health (RR-00542).

Received for publication 5 June 1981 and in revised form 24 March 1982.

REFERENCES

1. Benz, W., G. C. K. Roberts, J. Feeney, and R. R. Ison. 1972. Proton spin lattice relaxation studies of the histidine residues of pancreatic ribonuclease. *Biochim. Biophys. Acta.* 278:233–238.
2. Wasylishen, R. E., and J. S. Cohen. 1974. Spin-lattice relaxation times of imidazole protons and their relevance to NMR studies of proteins. *Nature (Lond.).* 249:847–850.
3. Kalk, A., and H. J. C. Berendsen. 1976. Proton magnetic relaxation and spin diffusion in proteins. *J. Magn. Reson.* 24:343–366.
4. Coates, H. B., K. A. McLauchlan, I. D. Campbell, and C. E. McColl. 1973. Proton spin lattice relaxation time measurements at 90 MHz and 270 MHz. *Biochim. Biophys. Acta.* 310:1–10.
5. Sykes, B. D., W. E. Hull, and G. H. Snyder. 1978. Experimental evidence for the role of cross relaxation in proton nuclear magnetic resonance spin lattice relaxation time measurements in proteins. *Biophys. J.* 21:137–146.
6. Andree, P. J. 1975. Magnetic resonance studies on glutamate dehydrogenase. Ph. D. Thesis. University of Groningen.
7. Andree, P. J. 1978. The effect of cross relaxation on the longitudinal relaxation times of small ligands binding to macromolecules. *J. Magn. Reson.* 29:419–431.
8. Arata, Y., R. Khalifah, and O. Jardetzky. 1973. NMR relaxation studies of the unfolding and refolding of staphylococcal nuclease at low pH. *Ann. N. Y. Acad. Sci.* 222:230–239.
9. Edzes, H. T., and E. T. Samulski. 1977. Cross-relaxation and spin diffusion in the proton NMR of hydrated collagen. *Nature (Lond.).* 265:521–523.
10. 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.
11. 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.
12. Eisenstadt, M., and M. E. Fabry. 1978. NMR relaxation in the hemoglobin-water proton spin system in red blood cells. *J. Magn. Reson.* 29:591–597.

13. Eisenstadt, M. 1981. NMR relaxation of protein and water protons in methemoglobin solutions. *Biophys. J.* 33:469–474.

14. Fermi, G. 1975. Three-dimensional Fourier synthesis of human deoxyhaemoglobin at 2.5 Å resolution: refinement of the atomic model. *J. Mol. Biol.* 97:237–256.

15. Ho, C., and I. M. Russu. 1978. Proton nuclear magnetic resonance studies of sickle cell hemoglobin. Biochemical and clinical aspects of hemoglobin abnormalities. W. C. Caughey, editor. Academic Press, Inc., New York. 179–193.

16. Russu, I. M., and C. Ho. 1980. Proton longitudinal relaxation investigation of histidyl residues of normal human adult and sickle deoxyhemoglobin: evidence for the existence of pregelation aggregates in sickle deoxyhemoglobin solutions. *Proc. Natl. Acad. Sci. U. S. A.* 77:6577–6581.

17. Russu, I. M. 1979. A proton nuclear magnetic resonance investigation of human normal adult and sickle hemoglobins. Ph.D. Dissertation. University of Pittsburgh.

18. Lindstrom, T. R., and C. Ho. 1972. Functional nonequivalence of the α and β hemes in human adult hemoglobin. *Proc. Natl. Acad. Sci. U. S. A.* 69:1707–1710.

19. Willard, J. M., J. J. Davis, and H. G. Wood. 1969. Phosphoenolpyruvate carboxytransphosphorylase. IV. Requirement for metal cations. *Biochemistry.* 8:3137–3144.

20. Pearson, H., D. Gust, I. M. Armitage, H. Huber, J. D. Roberts, R. E. Stark, R. R. Vold, and R. L. Vold. 1975. Nuclear magnetic resonance spectroscopy: reinvestigation of carbon-13 spin-lattice relaxation time measurements of amino acids. *Proc. Natl. Acad. Sci. U. S. A.* 72:1599–1601.

21. Russu, I. M., N. T. Ho, and C. Ho. 1980. Role of the β146 histidyl residue in the alkaline Bohr effect of hemoglobin. *Biochemistry.* 19:1043–1052.

22. Wüthrich, K. 1976. NMR in biological research: peptides and proteins. North Holland Publishing Company, Amsterdam.

23. Johnson, M. E., L. W.-M. Fung, and C. Ho. 1977. Magnetic field and temperature induced line broadening in the hyperfine-shifted proton resonance of myoglobin and hemoglobin. *J. Amer. Chem. Soc.* 99:1245–1250.

24. Bloembergen, N. 1949. On the interaction of nuclear spins in a crystalline lattice. *Physica.* 15:386–426.

25. Burton, D. R., S. Forsen, G. Karlstrom, and R. A. Dwek. 1979. Proton relaxation enhancement (PRE) in biochemistry: a critical survey. *Prog. NMR Spectrosc.* 13:1–45.

26. Abragam, A. 1961. The Principles of Nuclear Magnetism. Oxford University Press, Oxford, England. 139.

27. Solomon, I. 1955. Relaxation processes in a system of two spins. *Phys. Rev.* 99:559–565.

28. de Gennes, P. G. 1958. Sur la relaxation nucleaire dans les cristaux ioniques. *J. Phys. Chem. Solids.* 7:345–350.

29. Blumberg, W. E. 1960. Nuclear spin-lattice relaxation caused by paramagnetic impurities. *Phys. Rev.* 119:79–84.

30. Takahashi, S., A. K.-L. C. Lin, and C. Ho. 1980. Proton nuclear magnetic resonance studies of hemoglobin M Boston (α58E7His→Tyr) and M Milwaukee (β67E11Val→Glu): Spectral assignments of hyperfine-shifted proton resonances and of proximal histidine (E7) NH resonances to the α and β chains of normal human adult hemoglobin. *Biochemistry.* 19:5196–5202.

31. Woessner, D. E. 1962. Spin relaxation processes in a two-proton system undergoing anisotropic reorientation. *J. Chem. Phys.* 36:1–4.

32. Russu, I. M., N. T. Ho, and C. Ho. 1982. A ^{1}H NMR investigation of the role of histidyl residues in the alkaline Bohr effect of normal human adult hemoglobin. Hemoglobin and Oxygen Binding. C. Ho, W. A. Eaton, J. P. Collman, Q. H. Gibson, J. S. Leigh, Jr., E. Margoliash, J. K. Moffat, and W. R. Scheidt, editors. Elsevier North-Holland, Inc., New York. 141–149.