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Rotational and Translational Water Diffusion in the Hemoglobin Hydration Shell: Dielectric and Proton Nuclear Relaxation Measurements

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ABSTRACT The dynamic properties of water in the hydration shell of hemoglobin have been studied by means of dielectric permittivity measurements and nuclear magnetic resonance spectroscopy. The temperature behavior of the complex permittivity of hemoglobin solutions has been measured at 3.02, 3.98, 8.59, and 10.80 GHz. At a temperature of 298 K the average rotational correlation time τ of water within a hydration shell of 0.5-nm thickness is determined from the activation parameters to be 68 ± 10 ps, which is 8-fold the corresponding value of bulk water. Solvent proton magnetic relaxation induced by electron-nuclear dipole interaction between hemoglobin bound nitroxide spin labels and water protons is used to determine the translational diffusion coefficient $D(T)$ of the hydration water. The temperature dependent relaxation behavior for Lamor frequencies between 3 and 90 MHz yields an average value $D(298K) = (5 \pm 2) \times 10^{-10} m^2 s^{-1}$, which is about one-fifth of the corresponding value of bulk water. The decrease of the water mobility in the hydration shell compared to the bulk is mainly due to an enhanced activation enthalpy.

INTRODUCTION

The dynamics, and correspondingly the function of an enzyme, is strongly influenced by its surrounding. Lysozyme, for example, has no enzymatic activity as a dry protein (Rupley et al., 1980). Studies of protein dynamics by measurements of IR spectroscopy (Careri et al., 1980), Mößbauer spectroscopy (Goldanskii and Krupyanskii, 1989), and EPR spectroscopy (Steinhoff et al., 1989) have shown that the interaction of the protein surface with water molecules strongly increases the intramolecular mobility compared to the dry protein. The hierarchial order of substates and motions of a protein with the complicated dependence of the dynamics on hydration (Frauenfelder and Gratton, 1986) is evidence for the complex interaction of the protein with the solvent. The dynamics of the water molecules near the protein surface is influenced by protein-water interaction as well. Dynamic aspects of hydration are specified by the type and rate of motion of the water molecules. Three types of motion should be distinguished: proton exchange processes which limit the lifetime of a water molecule as a unit, translational motions and rotational motions. The lifetime of a water molecule at room temperature is in the order of milliseconds and since rotational and translational lifetimes in macromolecular hydrates are several orders of magnitude shorter, it is meaningful to consider the dynamics of hydration water in terms of these two processes.

Several approaches have been made in the past to study the rotational and translational motion of water molecules in the vicinity of the protein surface. By means of dielectric relaxation spectroscopy the rotational diffusion processes in aqueous protein solutions have been characterized (Buchanan et al., 1952; Schwan, 1957; Pennock and Schwan, 1969; Grant et al., 1978; Grant et al., 1986; Dachwitz et al. 1989). Between three and five relaxation contributions have been separated in the frequency region between 100 kHz and 72 GHz. It is agreed that the relaxation below 10 MHz (β relaxation) originates from the tumbling of the whole protein molecule, while the relaxation around 20 GHz (γ relaxation) is due to the rotational diffusion of the bulk water. Recent measurements give evidence that a second relaxation process is needed to describe the dielectric behavior of bulk water found in the frequency range above 20 GHz (Dachwitz et al., 1989; Zaghloul and Buckmaster, 1985). In the intermediate frequency range, between 15 MHz and 10 GHz, a weak δ process is measurable, which is now assumed (Pennock and Schwan, 1969; Grant et al., 1978; Grant et al., 1986; Dachwitz, et. al 1989) to consist of contributions from side chain fluctuations and rotational diffusion of water molecules at the protein surface. The water involved has been referred to as "bound water" or "water of hydration." The respective magnitudes and correlation times of the electric dipole moment fluctuations due to side chain motions and water rotational diffusion at the protein surface, however, are still subject to discussions.

The dielectric behavior of bound water cannot be directly investigated. Any information about this system has to be inferred from a study of the difference in behavior of the solution and the solvent. In an investigation of the permittivity of bound water, the well known experimental problem of measuring the difference between two large quantities is therefore encountered. Recent developments, including methods and equipment in our laboratory, provide the means to perform permittivity measurements of solutions with uncertainties in the 1% range, and it seems worthwhile to reinvestigate the dielectric behavior of bound water in protein solutions (Zaghloul and Buckmaster, 1985; Redhardt et al., 1989; Steinhoff et al., 1990; Barthel et al., 1992). We have

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performed temperature-dependent measurements of the dielectric permittivity of hemoglobin solutions in the frequency range between 3 and 11 GHz. These measurements provide information about the rotational diffusion of the water molecules and the amount of water influenced by interactions with the protein surface.

Additionally, the localized translational diffusion of water molecules near the protein surface is measured by means of NMR spectroscopy. The results are compared to the translational self diffusion of free water. For this purpose nitroxide radical side chains are covalently attached to hemoglobin at different surface sites. The paramagnetic center induces solvent proton relaxation, and the dominance of translational contributions to the correlation time for the electron-nucleus interaction permits the characterization of the translational diffusion of the solvent in local environment of the paramagnetic center (Polnaszek and Bryant, 1984). Thermodynamic parameters of the rotational and translational diffusion processes of the hydration water are presented and compared to the respective values of free water.

EXPERIMENTAL PROCEDURES

Horse methemoglobin (methb) was prepared from fresh blood samples. The cell suspensions were centrifuged 15 min at 2500 *g*, then washed three times with 0.9% NaCl solution and hemolyzed by addition of equal amounts of distilled water. To separate the membranes the samples were frozen in liquid nitrogen and centrifuged, after thawing, at 30,000 *g* for 1 h. A twofold excess of $K_3[Fe(CN)_6]$ was added to oxidize the oxyhemoglobin to methemoglobin. For the permittivity measurements salts were removed by dialysis against distilled water for 12 h. methb concentrations were determined spectroscopically as methbCN using an extinction coefficient $\epsilon_{540\ nm}$ = 11,500 liters/(mol cm). The samples were diluted with distilled water to a concentration of 6.5 mM. The conductivity σ' of the samples was determined using a homemade 1-kHz bridge; σ' values of 800 μS/cm have been measured. After the dialysis step this value remains stable within a 5% limit for several hours.

For the NMR measurements spin labeling of oxyhemoglobin followed the procedure of McCalley et al. (1972). The spin label *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)maleimide (MalSl) is covalently bound to the cysteine β93 (hbMSl). The nitroxide tip of MalSl occupies a hydrophobic pocket of the hemoglobin molecule providing a label position within the surface of the protein (Moffat, 1971). Lysines of oxyhemoglobin were labeled by incubation for 5 h with *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)-iodoacetamide (IaaSl) in borate buffer at pH 10 and 298 K (hblysSl). The cysteine β93 had been previously blocked by iodoacetamide. In this case EPR spectroscopy study has shown that the motion of the spin label side chain is less hindered by neighboring side chain atoms, and the location of the spin label tip was assumed to be at the surface of the hemoglobin molecule (Steinhoff, 1990). The modified oxyhemoglobins were oxidized to methb as described above. The samples were desalted and made free from unbound spin labels by passing them through a column of Sephadex G-25. For NMR measurements the methb concentration in 0.1 M phosphate buffer of pH 6.8 is adjusted to 1.2 mM. Concentrations of the attached spin labels were determined from the comparisons of the double-integrated EPR spectra with that of reference samples, of which the spin number has been measured by an absolute method (Redhardt and Daseler, 1987).

The dielectric permittivity of pure water and hemoglobin solutions were measured at four discrete frequencies in a range between 3 and 11 GHz using transmission cells and a homodyne detection method recently described in detail elsewhere (Steinhoff et al., 1990). The instrument was built in this laboratory and makes possible the measurement of the permittivities of water and aqueous solutions with uncertainties in the 1% range. The temperature of the isolated transmissions cells was held constant using a temperature controlled water heat reservoir. The temperature values of the samples were measured during the data collection with thermocouples which have been calibrated by mercury thermometers with authenticated accuracies of ± 0.01°C. The relative temperature drift during a measurement was less than 0.008°C.

NMR measurements of the solvent proton relaxation were performed with a conventional pulse spectrometer B-KR-322s (Bruker, Rheinstetten, Germany) equipped with two 12-bit AD converters (Analog Devices, Norwood, MA), external pulse generators (homemade), and a BST-100-700 temperature control unit (Bruker). Pulse control, data recording, and data processing were done by two connected personal computers (Atari 1040 ST; Atari GmbH, Raunheim, Germany). Details of the apparatus and the control units were described by Kramm (1992). Four discrete field strengths corresponding to proton resonance frequencies ranging from 3 to 90 MHz have been used. The free induction decay signals after 180–τ–90 pulse sequences were accumulated to achieve an appropriate signal-to-noise ratio, Fourier-transformed, phase-corrected, and integrated. Due to the large differences in T_2 of protein protons and water protons the relaxation of the protein protons do not significantly contribute to the signal in the present spectrometer settings. The relaxation rates for the water protons were obtained from a least squares fit of a single exponential to the data obtained from 15 τ values. Differences between the experimental recovery curves and the fitted single exponentials did not show any systematic deviation.

THEORY

Dielectric relaxation

The permittivity is a complex quantity $\epsilon^* = \epsilon' - i\epsilon''$, of which the real part ϵ' represents the ratio of the in-phase component of the electric displacement D' to the electric field E, and the imaginary part ϵ'' represents the 90° out-of-phase component of D'. The frequency dependence of the contribution to the dielectric constant from the dipole moments of the water molecules is directly related to the Fourier transform of the correlation function $\langle m_z(t)m_z(t+\tau)\rangle$ of the dipole moment of a large assembly of molecules, where $m_z(t)$ is the time-dependent component of this dipole moment in the direction of the applied field. The dielectric behavior of water may be adequately described by a relation of the Cole-Cole type (Cole and Cole, 1941),

$$\epsilon^* = \epsilon_\infty + (\epsilon_s - \epsilon_\infty)/[1 + (i\omega\tau_d)^{1-\alpha'}], \quad (1)$$

or of the Debye type with the empirical parameter α' set to zero (Kaatze, 1989). The correlation function in this Debye case is exponential:

$$\langle m_z(t)m_z(t+\tau)\rangle = \langle m_z^2\rangle \exp(-\tau/\tau_d). \quad (2)$$

ϵ_∞ and ϵ_s are the high and low frequency limits of the dielectric permittivity, respectively, and τ_d is the relaxation time. The empirical parameter α' of the Cole-Cole equation accounts for a possible narrow distribution of relaxation times. The dielectric relaxation time is not equal to the correlation time of the single molecular dipole moments, because in liquids the dipole moment of any molecule interacts with the dipole moments of its neighbors and the time dependence of the dipole moment of an assembly of molecules is not simply determined by the time dependence of the statistical sum of individual molecular dipole moments. The exact description of this process depends on the detailed mo-

lecular model. However, the difference between dielectric relaxation time and molecular correlation time has been estimated to be less than 15% (Bordewijk, 1973; Sedykh and Feldman, 1977), so that the macroscopic value will be used to characterize the rotational motion of the water molecules.

An aqueous solution of macromolecules is heterogeneous with respect to the conductivity and permittivity of the individual components of the system. A macromolecule with a low permittivity and conductivity is surrounded by aqueous layers with possibly different permittivities and conductivities. The physical model and mathematical means for the interpretation of the permittivity measurements of such a system will be given and discussed in Results.

Nuclear magnetic resonance

The nuclear spin relaxation times of the water protons in a solution of paramagnetic nitroxide spin labels are influenced by the intermolecular dipolar interaction between the unpaired electron (the S spin) on the nitroxide and the nuclear proton spins (the I spin) on water. As shown by Borah and Bryant (1981) and Polnaszek and Bryant (1984) the modulation of the I-S dipolar interaction due to the relative translational diffusion of the I and S spin containing molecules is the dominant process in these systems. For S = ½ spins the general form for the relaxation equation in this case is (Abragam, 1970)

$$T_1^{-1} = \frac{\pi\gamma_I^2\gamma_S^2\hbar^2}{16}[J(\omega_S - \omega_I) + 3J(\omega_I) + 6J(\omega_I + \omega_S)], \tag{3}$$

where γ_I and γ_S are the nuclear and electron magnetogyric ratios, $\hbar$ is Planck's constant, and ω_I and ω_S are the Lamor frequencies of the nuclear and electron spin for the applied field. The IS dipolar contribution to the spectral densities J is model-dependent (Polnaszek and Bryant, 1984; Hubbard, 1965; Ayant et al., 1975; Hwang and Freed, 1975). In the analysis of our data we use different models which all include the effect of the finite size of the diffusing particles. The first model to account for translational diffusion in solutions was developed by Torrey and is based on the theory of random flight (Torrey, 1953). The spectral densities $J(\omega)$ were given as a function of α, the ratio of the mean square flight path for a single diffusion step $\langle r^2\rangle$ to the distance of closest approach d,

$$\alpha = \langle r^2\rangle/(12d^2). \tag{4}$$

We use the formulas given by Krüger (1969),

$$J(\omega) = \frac{32n_S x_I}{5x^2d^3\omega_I}(1/5 + \alpha) \times \{v(1 - w) + [v(1 + w) + 2]\exp(-2v)\cos(2u) + u(1 - w)\exp(-2v)\sin(2u)\} \tag{5}$$

$$w = \frac{1}{(u^2 + v^2)} \tag{6}$$

$$\frac{v}{u} = \frac{\sqrt{q(1 \pm q)}}{2\sqrt{\alpha}} \tag{7}$$

$$q = \left[1 + \frac{1}{x^2}\sqrt{1 + \frac{1}{5\alpha}}\right]^{-1/2} \tag{8}$$

$$x_{I,S} = \omega_{I,S}\tau_c, \tag{9}$$

where the correlation time τ_c is given by

$$\tau_c = \frac{d^2/5 + \langle r^2\rangle/12}{D} \tag{10}$$

and D is the relative translational diffusion coefficient

$$D = D_I + D_S \tag{11}$$

where D_I and D_S are the individual diffusion constants of the molecules containing the I and S spins, respectively, and n_s (Eq. 5) is the S spin density. The two other models to be applied start from the diffusion equation for continuous isotropic translational diffusion to calculate the correlation function. In the treatment by Hubbard (1965) the finite size of the particles and therefore the distance of closest approach d is accounted for by a lower integration boundary in the calculation of the correlation function. The spectral density has been derived in closed form as follows

$$J(\omega) = \frac{16n_S}{5dDy^5}\{y^2 - 2 + \exp(-y) \times [(y^2 - 2)\sin(y) + (y^2 + 4y + 2)\cos(y)]\} \tag{12}$$

with

$$y = (\omega\tau)^{1/2}. \tag{13}$$

The translational correlation time τ is

$$\tau = d^2/D. \tag{14}$$

The model which includes the effect of closest approach already in the solution of the diffusion equation is the so called force-free model (Ayant et al., 1975; Hwang and Freed, 1975) for which the spectral density function is given by

$$J(\omega) = \frac{128n_S}{135dD} \times \frac{1 + 5z/8 + z^2/8}{1 + z + z^2/2 + z^3/6 + 4z^4/81 + z^5/81 + z^6/648} \tag{15}$$

with

$$z + (2\omega\tau)^{1/2} \tag{16}$$

These three models will be used in the next section to explain the experimental data.

RESULTS AND DISCUSSION

The permittivity of water and hemoglobin solutions

To separate the different contributions of free water and bound water to the effective permittivity of a protein solution we have first analyzed the permittivity of pure water and give the means to calculate $\epsilon^*(T, \omega)$ for intermediate temperature and frequency values. For this reason the permittivity $\epsilon^* = \epsilon' - i\epsilon''$ of distilled water has been determined for four frequencies, ν = 3.02, 3.98, 8.59, and 10.80 GHz, in the temperature range between 5° and 50°C. To describe the water relaxation we use the Cole-Cole equation (Eq. 1). To account for the temperature dependence of ϵ^* the following expressions are used (Vidulich et al., 1967; Hill et al., 1969)

$$\epsilon_S(T) = \epsilon_S\,(0°C)\exp(\beta T) \tag{17}$$

$$\tau(T) = \frac{h}{kT}\exp\left(\frac{\Delta H^{\#}}{RT} - \frac{\Delta S^{\#}}{R}\right), \tag{18}$$

where $\Delta H^{\#}$ and $\Delta S^{\#}$ are the molar enthalpy and entropy of activation, respectively. The static contribution of the permittivity, ϵ_S, and its temperature dependence have been measured with high precision (Vidulich et al., 1967). Therefore, following Steinhoff et al. (1990), literature values for β and ϵ_S (0°C) have been used, and the remaining four parameters $\Delta H^{\#}$, $\Delta S^{\#}$, α, and ϵ_∞ (cf. Eq. 1) have been determined from the set of experimental values by means of a least squares fit procedure. The experimental results and the curves calculated from the best fit parameters are shown in Fig. 1. The parameters are in agreement with our previous measurements determined on the basis of only two frequencies (Steinhoff et al., 1990, Table 4). The accuracy has been improved: The average absolute standard deviation of the data points from the fitted curves σ_{tot} is 0.26 compared to 0.34 in Steinhoff et al. (1990). The calculated curves fit the experimental data very well (Fig. 1). The slight difference between experiment and theory may be due to the use of an average temperature-independent $\Delta H^{\#}$. This has provided a reasonable, but perhaps not entirely complete description of the variation of τ with temperature. In fact, least squares fits of a single-τ Debye type model to the experimental data points in limited temperature ranges yield values of $\Delta H^{\#}$ of (17.3 ± 0.1) kJ/mol in the range 5°C ≤ T ≤ 30°C, (16.94 ± 0.08) kJ/mol in the range 5°C ≤ T ≤ 40°C, and (16.65 ± 0.08) kJ/mol in the range 5°C ≤ T ≤ 50°C. This results indicates a systematic decrease of the average activation enthalpy with increasing temperature.

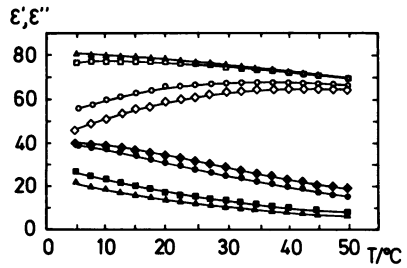

FIGURE 1 Dielectric permittivity, ϵ'(T) (open symbols) and loss ϵ''(T) (closed symbols), of water at four frequencies. The solid lines are calculated with a single-τ Debye model using the parameters of free water (Table 1). (△), ν = 3.02 GHz; (□), ν = 3.98 GHz; (○), ν = 8.59 GHz and (◇), ν = 10.8 GHz.

The measurements of ϵ' and ϵ'' for the hemoglobin solutions are performed over the temperature range 5 to 25°C. As the parameters of the free water relaxation are needed in the analysis of the results for the hemoglobin solutions and these parameters seem to be temperature-dependent, they are also determined from fits of a single-τ Debye type model to the experimental data in the temperature range 5 to 30°C. The best fit parameters for this temperature range are given in Table 1, a standard deviation σ_{tot} of 0.2 is calculated. The inclusion of a nonzero α does not further improve the fits.

The value of ϵ_∞ determined from our single-τ Debye type approach does not coincide with the values expected from FIR data in the 170–410-GHz region (Hasted et al., 1987). This result does not significantly depend on the upper temperature limit of the fits and is also true for a Cole-Cole approach with an additional fit parameter α. To account for this discrepancy we have to assume a further dispersion in the frequency region above 20 GHz. Evidence for a fast relaxation process in the 30–100-GHz region has also been given by Dachwitz and coworkers (1989) and by Barthel et al. (1992). The latter authors suggest that the rotation of water molecules within the so-called asymmetrical two-bonded state with one donor and one acceptor site of the molecule involved in hydrogen bonds is responsible for the relaxation with a characteristic time of 1 ps.

The parameters given in Table 1 are now used to calculate the values of the dielectric permittivity of water, $\epsilon_w^*(T, \omega)$, for intermediate values of the temperature T and frequency ω for the studies in the next section.

The results of our dielectric permittivity measurements of methemoglobin solutions are shown in Fig. 2. The differences $\Delta\epsilon^*$ between the calculated values of water, ϵ_w^*, and the experimental values of the protein solutions, ϵ^*, scaled to a concentration of hemoglobin of 1 g/liter, the so-called specific dielectric decrements, are given for hemoglobin concentrations ranging from 6.0 to 6.5 mM (i.e., 102–110 g/liter). The absolute mean standard deviations of the experimental data points are calculated to be 0.004 liter/g for $\sigma(\Delta\epsilon'/c)$ and 0.002 liter/g for $\sigma(\Delta\epsilon''/c)$. The electrical conductivity, σ', of the hemoglobin solutions has been minimized by dialysis of the solution against distilled water. However, the influence of the resulting value of σ' of 800 μS/cm cannot be neglected for the frequency range below 5 GHz. According to the Maxwell equations a generalized permittivity results for conducting samples, which is written

$$\epsilon_w^* = \epsilon_w' - i[\epsilon_w'' + \sigma'/(\epsilon_0\omega)]. \tag{19}$$

TABLE 1 Kinetic parameters and permittivity data of free water and water within the hydration shell of hemoglobin

Type of water	$\Delta S^{\#}/R$	$\Delta H^{\#}$	ϵ_∞	ϵ_s (0°C)	β	b	τ (298K)
		kJ mol^{-1}			°C^{-1}	g^{-1} ml	ps
Free	2.69 ± 0.04	17.3 ± 0.1	6.7 ± 0.2	87.91*	0.00458*		8.5 ± 0.3
Bound*	3.8 ± 1.4	24.4 ± 3.4	3.4	87.91*	0.00458*	0.29 ± 0.01	68 ± 10
Bound‡	2.8 ± 1.5	22.3 ± 3.5	3.4	91.5‡	0.00287	0.28 ± 0.01	80 ± 10

The values of the kinetic parameters and b are determined from least squares fits of Eqs. 1 and 17–22 to the experimental data points of pure water (Fig. 1) and of hemoglobin solutions (Fig. 2), respectively. The values for bound water have been determined with fixed values of ϵ_∞ (see text) and applying two different approaches for the static dielectric constant:

*Values of the static dielectric constant, ϵ_s (0°C) and β, have been fixed according to the values of liquid water (Vidulich et. al., 1967).

‡Values of ϵ(0°C) and β have been fixed according to the values of ice (Auty and Cole, 1952).

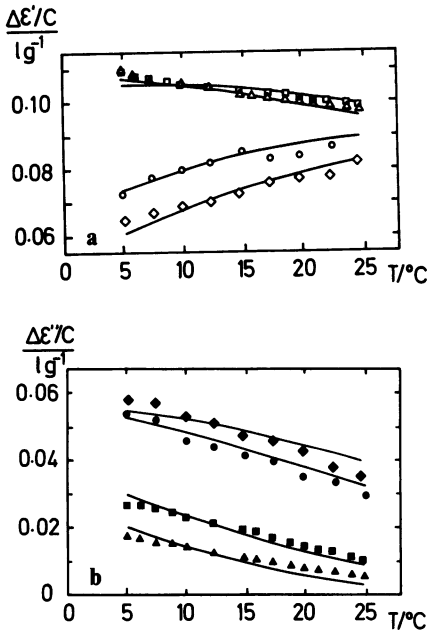

FIGURE 2 Temperature dependence of the specific dielectric decrement, $\Delta\epsilon^*/c = (\epsilon_w^* - \epsilon^*)/c$. The real part, $\Delta\epsilon'/c$ is given by open symbols. (△) ν = 3.02 GHz; (□) ν = 3.98 GHz; (○) ν = 8.59 GHz; and (◇) ν = 10.8 GHz. The absolute error limits of the experimental data points are 0.004 liter/g for $\Delta\epsilon'/c$ and 0.002 liter/g for $\Delta\epsilon''/c$. The solid lines are calculated by means of the Maxwell-Wagner mixing equations with a single-τ Debye model for the bound water relaxation. The parameters given in Table 1, second row, are used.

The conductivities of the present samples have been taken into consideration for the differences $\Delta\epsilon^*/c$ shown in Fig. 2.

To account for the measured behavior of the permittivity and to separate the possible contributions to the dielectric relaxations we proceed as described by Pennock and Schwan (1969). The contribution of the overall tumbling of the hemoglobin molecule to the relaxations above 100 MHz is negligible. Measurements of the rotational correlation times of spin label side chains attached to different locations at the protein surface suggest that a dispersion for side chains has a maximum characteristic frequency of about 160 MHz at physiological temperatures (Steinhoff et al., 1989; Steinhoff, 1991). So the remaining contributions to the relaxation are considered to originate from the hydration shell near the protein surface. We apply a macroscopic physical model of the hemoglobin solution consisting of particles with a protein core surrounded by a bound water shell suspended in an electrolyte medium. The measured dielectric permittivity is related to the permittivity of the protein core, the water shell and the surrounding electrolyte by a pair of mixture equations (Wagner, 1914). For a dilute solution of spherical molecules these equations written in complex form are

$$\frac{\epsilon^* - \epsilon_w^*}{\epsilon^* + 2\epsilon_w^*} = q\,\frac{\epsilon_h^* - \epsilon_w^*}{\epsilon_h^* + 2\epsilon_w^*} \tag{20}$$

and

$$\frac{\epsilon_h^* - \epsilon_b^*}{\epsilon_h^* + 2\epsilon_b^*} = \left(\frac{R}{R+d}\right)^3 \frac{\epsilon_p^* - \epsilon_b^*}{\epsilon_p^* + 2\epsilon_b^*} \tag{21}$$

where R is the protein radius and d is the thickness of the bound water shell. The subscripts w, h, p, and b denote the external electrolyte, the hydrated hemoglobin molecule, the dry protein, and the bound water shell, respectively, and q is the volume fraction of the total solute which is occupied by the hydrated hemoglobin particles. The quantity $R/(R + d)$ is calculated in terms of the specific volume v_p of hemoglobin (v_p = 0.75 ml/g) and the amount of bound water $b = v_b m_b/m_p$

$$[R/(R+d)]^3 = v_p/(v_p + b) \tag{22}$$

m_p and m_b are the masses of the protein and the bound water, respectively. v_b is the specific volume of water in the hydration shell and is set to 1 ml/g. The fraction q may also be expressed in terms of the specific volumes and the concentration c of the protein, $q = c(v_p + b)$.

The dielectric constant of the hemoglobin core is assumed to be about the same as that of dried hemoglobin powder, i.e., ϵ_p = 2.3 (Postow and Rosenberg, 1970). To account for the frequency and temperature dependence of the dispersion of the water in the hydration shell, ϵ_b^*, Eqs. 1, 17, and 18 are assumed to hold. For the purpose of the present discussion we assume that the relaxation in the hydration shell is characterized in terms of a single-τ Debye function so that α = 0. Values of $\epsilon_S^*(T)$ determined for bulk water according to the results given by Vidulich and coworkers (1967) and for ice according to the results of Auty and Cole (1952) have been used to cover the range of physically meaningful values of the low frequency limit value of $\epsilon_b^*(T,\omega)$, ϵ_{bs} (T). The respective values of ϵ_S (0°C) and β are given in Table 1. Three parameters remain to be fitted: $\Delta S^{\#}$, $\Delta H^{\#}$ and b.

The results of our calculations with $\epsilon_{bs}(T)$ fixed to the the value of bulk water, $\epsilon_{S,water}(T)$, are shown in Fig. 2. The values of the best fit parameters for this model as well as for the calculations with $\epsilon_{bs}(T)$ fixed to the value of ice are given in Table 1. The calculated behavior of $\Delta\epsilon^*(T)/c$ is in good agreement with the experiment data, if we consider the error limits of the data points given above. Variations of the high frequency limit value ϵ_∞ from 3.4 to 6.7 did not change the quality of the fits nor did the fit parameters differ significantly from the values given in Table 1. In spite of the very different temperature dependencies assumed for $\epsilon_{bs}(T)$ the values of the fitted parameters are identical within their error limits. The resulting hydration value $b = 0.29$ g of water per g of hemoglobin is in good agreement with values given by other authors (Pennock and Schwan, 1969; Grant et al., 1978). The thermodynamic data yield a rotational correlation time of bound water which is about 8-fold the value of τ of free water for physiological temperatures.

NMR measurements of the translational diffusion of water in the hydration shell

The NMR method for the determination of the translational diffusion properties of water in the vicinity of a protein surface is first tested with a simple system. The water proton relaxation rates for a 0.1 M phosphate buffer solution containing the low molecular weight nitroxide TEMPO (*N*-(1-oxy-2,2,6,6-tetramethylpiperidine)), have been measured as a function of temperature. Four field strengths according to Lamor frequencies of 3, 13, 50, and 90 MHz have been used. Since the limit of fast exchange of water molecules between compartments close to the nitroxide and the bulk may be assumed, the measured relaxation rates of nitroxide free buffer solution have been subtracted, the results are shown in Fig. 3. To analyze the data spectral densities according to Eqs. 12 or 15 are used. The temperature dependence of the diffusion coefficient D is accounted for by an Arrhenius expression,

$$D = D_0 \exp(-\Delta H_t^{\#}/RT). \quad (23)$$

The agreement between theory and experiment is very good in both cases (Eqs. 12 and 15), curves calculated from the best fit parameters of Eqs. 15 or 12 to the experimental data points almost coincide, so only the curves calculated according to Eq. 12 are shown in Fig. 3. The fit parameters for both models are given in Table 2. The slight difference between theory and experiment, which is obvious for the low temperature data shown in Fig. 3, might be caused by an additional relaxation contribution due to the rotational motion of the complex of the nitroxide and a water molecule. It was shown earlier that NMR relaxation dispersion data have increasing rotation character as the temperature is lowered (Borah and Bryant, 1981). However, inclusion of this rotational contribution changes the calculated translational diffusion coefficient only little (Polnaszek and Bryant, 1984).

The diffusion coefficient of the relative translational diffusion of water and the nitroxide of the present sample is calculated from the fitted parameters. For the force-free model (Eq. 15, Table 1, row 1) we find $D = (1.9 \pm 0.2) \times 10^{-9}$ m^2 s^{-1} at $T = 300$ K, the application of Eq. 12 gives a diffusion coefficient of half this value. Assuming that the diffusion of the nitroxide obeys Stokes law a self diffusion coefficient of water at the nitroxide of $(1.6 \pm 0.2) \times 10^{-9}$ m^2 s^{-1} is determined in the present buffer solution applying the force-free model. If we correct this value according to the increased viscosity of the solution due to the buffer ions the resulting value is about 30% less than the known value for pure water of 2.5×10^{-9} m^2 s^{-1} (Hrovat and Wade, 1980). We conclude that the agreement between theory and literature values for the translational self diffusion of water is better for the force-free model compared to the model leading to Eq. 12. Therefore, we restrict ourselves to the discussion of the results on the basis of the force-free model in the remainder of the paper.

The distance of closest approach is in very good agreement with the results of Polnaszek and Bryant (1984). However, it is shorter than the value expected from the van der Waals radii of TEMPO (Lajzerowicz-Bonneteau, 1976) and water, which is $d \approx 0.5$ nm. This discrepancy is a consequence of locations of the spins off the centers of the molecules and of the present unavailability of a suitable pair correlation function for the TEMPO-water system. The value of the enthalpy of activation is in good agreement with the known bond energies of hydrogen bonds. The above results show the usefulness and limits of the method and we conclude that a combination of temperature-dependent and frequency-dependent measurements of the relaxation rates provides useful information about the translational diffusion of water.

In the limit of rapid exchange between free water and hydration water and assuming that the hydration water represents only a small fraction of the solvent, the effective proton relaxation rate T_{1eff}^{-1} of a dilute aqueous solution of spin labeled proteins is given by a sum of discrete

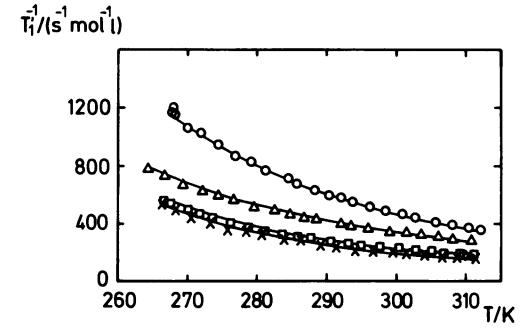

FIGURE 3 Electron-nuclear spin dipole interaction contribution to the water proton relaxation rates, the specific rate $T_{1n}'^{-1}$, of a TEMPO solution in 0.1 M phosphate buffer is shown for four Lamor frequencies. (○) ν = 3 MHz; (▲) ν = 13 MHz; (□) ν = 50 MHz; and (x) ν = 90 MHz. The solid line is calculated using the force-free model for isotropic translational diffusion (Eq. 15) with parameters given in Table 2.

Table 2 Translational diffusion parameters evaluated for bulk water and for water in the vicinity of the hemoglobin surface for different diffusion models

Sample	Model (Eq.)	D_0	$\Delta H_t^{\#}$	D (300K)	d	α
		$10^{-5}m^2s^{-1}$	kJ/mol	$10^{-10}m^2s^{-1}$	nm	
TEMPO solution	15	2.2 ± 0.3	23.3 ± 0.3	19 ± 2	0.27 ± 0.03	
	12	1.0 ± 0.3	23.3 ± 0.3	8.6 ± 1.0	0.25 ± 0.03	
hblysSl	15	3.7 ± 1.5	27 ± 1	7.2 ± 4.2	0.15 ± 0.1	
	5	9 ± 2	30 ± 1	5.3 ± 1.2	0.18 ± 0.01	0.018
hbMSl	15	6 ± 3	29 ± 1	5.2 ± 3.7	0.13 ± 0.01	
	5	9 ± 3	31 ± 1	4.0 ± 1.8	0.15 ± 0.01	0.017

contributions

$$1/T_{1\text{eff}} = c_p/T'_{1p} + c_n/T'_{1n} + 1/T_{1\text{buffer}}, \qquad (24)$$

where c_p and c_n are the molar concentrations of the protein and the attached nitroxide spin label, respectively. $T'_{1p}{}^{-1}$ and $T'_{1n}{}^{-1}$ are specific relaxation rates. $T'_{1p}{}^{-1}$ represents the diamagnetic contribution of the protein which was determined separately from a solution of unlabeled methemoglobin. The frequency-dependent values of this contribution increase monotonically with increasing temperature from between 200 (at 90 MHz) and 400 liters mol^{-1} s^{-1} (at 3 MHz) for T = 267 K to between 300 (at 90 MHz) and 550 liters mol^{-1} s^{-1} (at 3 MHz) for T = 315 K. Third order polynomials were fitted to the experimental $T'_{1p}{}^{-1}$ data to describe the temperature behavior for 3, 13, 50, and 90 MHz in the temperature range given above. Then, the SI dipole interaction contribution $T'_{1n}{}^{-1}$ was calculated from the experimental data of the effective rates, $T_{1\text{eff}}{}^{-1}$, determined for solutions of the spin-labeled methemoglobins, and the known contributions of $T_{1\text{buffer}}{}^{-1}$ and c_p/T'_{1n}. As an example for the importance of the respective terms in Eq. 24, the experimental values determined for a hblysSl sample for 3 MHz and T = 290 K are given: $T_{1\text{eff}}{}^{-1}$= 2.6 s^{-1}, c_p/T'_{1p} = 0.44 s^{-1}, c_n/T'_{1n} = 1.8 s^{-1} and $T_{1\text{buffer}}{}^{-1}$ = 0.36 s^{-1}. The temperature and frequency dependence for $1/T'_{1n}$ of the hblysSl and hbMSl samples are shown in Fig. 4. The analysis of this data is performed in terms of the three translational diffusion models. The standard deviations calculated from the fits, as well as the fit parameters of the force free-model (Eq. 15) and the approach given by Hubbard (Eq. 12) are similar. However, the best fits are obtained with the jump diffusion model of Torrey (Eq. 5). The solid curves shown in Fig. 4 were calculated from the best fit parameters according to this model. The parameters obtained for the force-free model and the jump diffusion model are given in Table 2. The parameter α appears to be much less than unity which is evidence that the relaxation is dominated by a translational process (Krüger, 1969). Consequently, the addition of a rotational contribution could not further improve the fits. Again the distance of closest approach is less than the value expected from the sum of the van der Waals radii. No significant difference is found for the two hemoglobin samples, though the locations of the nitroxides with respect to the water shell were expected to be different. As in the case of the TEMPO solutions, relevant information cannot be drawn from the absolute value of this parameter because of the lack of a suitable pair correlation

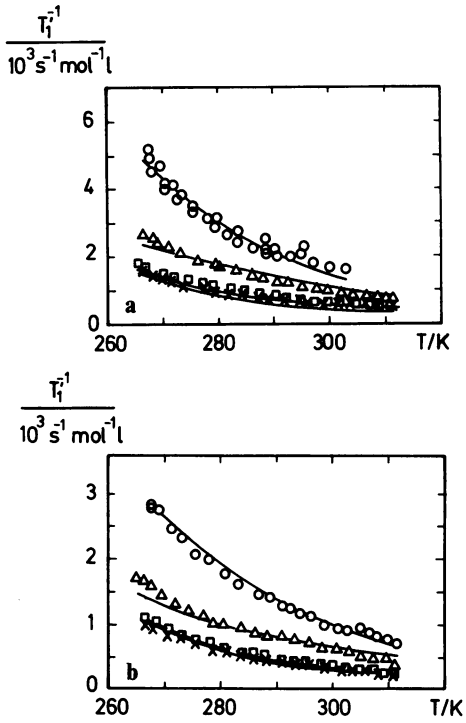

FIGURE 4 Electron-nuclear spin dipole interaction contribution to the water proton relaxation rates of spin-labeled methemoglobin solutions. The specific relaxation rate $T_{1n}{}^{-1}$, for four Lamor frequencies are represented by (○) ν = 3 MHz; (△) ν = 13 MHz; (□) ν = 50 MHz; and (x) ν = 90 MHz. The solid lines are calculated using the jump diffusion model, Eq. 5, with parameters given in Table 2. (*a*) MalSl spin label bound to cys β93; (*b*) IaaSl spin label bound to a surface lysine group.

function of the nitroxide-water interaction and the neglect of the effect on the relaxation rate due to the spins not being located at the centers of the interacting molecules. A third possible reason for the underestimation of d will be given below. The enthalpy of activation for the translational motion of water molecules in the vicinity of the protein surface is enhanced by about 20% compared to the value of free water. As in the case of the rotational diffusion, this behavior reflects the hydrogen bond interaction of the water molecules with certain surface atoms of the protein. In our two samples the nitroxides are covalently attached to two different sites of the protein. In the case of hbMSl the label occupies a hydrophobic pocket and the translational motion of the nitroxide is only due to the rotational and translational diffusion of the whole hemoglobin molecule. The corresponding value of the diffusion coefficient of the nitroxide is much

smaller than the values given in Table 2. Even if the nitroxide is able to perform residual motions at the protein surface, as it is assumed for the nitroxide in the hblysSl samples, the values of the resulting diffusion coefficient are significantly less (Steinhoff et al., 1991). Therefore the value of D derived from the fits is that appropriate to the water in the vicinity of the nitroxide at the protein surface. The analysis of the distance dependence of the SI interaction described by Polnaszek and Bryant (1984) indicates that these diffusion coefficients are an average over such coefficients of the water molecules within the first 1 nm of the nitroxide moiety.

In the present treatment we have neglected the contribution to the water proton relaxation induced by changes in the protein proton relaxation rate caused by the presence of the spin label. The presence of the paramagnetic center in the protein increases the protein proton relaxation time which may indirectly affect the water relaxation rate. This error source is discussed by Polnaszek and Bryant (1984) and no means are presently seen to circumvent this problem. The consequence of this neglect is to overestimate the paramagnetic contribution to the solvent relaxation and consequently the value of the distance of closest approach has to be regarded as a lower bound, and the value of the diffusion coefficients given in Table 2 have to be regarded as upper bounds.

We conclude that the translational diffusion coefficient of water is about three to five times smaller at the protein surface compared to the corresponding value of free water. The experimental data do not differ significantly for the two different spin label binding sites. Thus, the value obtained is regarded as representative of the diffusion constant at several points on the surface.

The results of the dielectric permittivity and NMR measurements on hemoglobin suggest that the main fraction of water near the protein surface, the so-called bound water, is not at all immobilized. Our results are consistent with recent experimental and theoretical estimates of surface solvent mobility. Otting and Wüthrich (1989) have studied water molecules for bovine pancreatic trypsin inhibitor in solution using nuclear Overhauser effects (NOEs). A few water protons or protein hydroxyl groups at the protein surface had lifetimes longer than 300 ps, but most had shorter lifetimes because their NOEs were not observed. Halle and coworkers (1981) used water ^{17}O magnetic relaxation to study the rotational correlation time of water molecules in the hydration shell. Cross-relaxation, which contributes to ^{1}H relaxation as discussed above, is unimportant for ^{17}O quadrupolar relaxation. The authors found approximately two layers of hydration water having a rotational correlation time about 8-fold the value of bulk water.

A dielectric permittivity investigation of myoglobin solutions by Dachwitz et al. (1989) gives evidence for two dispersions with relaxations times of about 2.5 ns and 200 ps. The fast relaxation time of about 200 ps was assigned to the motion of bound water, while the relaxation in the nanosecond time region was suggested to involve internal motions of the myoglobin molecule. This value has to be compared to results on myoglobin reported by Grant and coworkers (1986): Besides relaxations in the low MHz region, a small dispersion centered around 4 GHz, i.e., at a frequency only a factor five lower than the relaxation frequency of the bulk water dispersion, was detected in myoglobin solutions. It was suggested that this dispersion is due to water of hydration. The 5-fold increase of τ for bound water compared to bulk water is very similar to the 8-fold increase reported in the present work (cf. Table 1). When compared to previous investigations (Pennock and Schwan, 1969; Grant et al., 1978) where the dispersion due to the presence of a hydration shell was found to be centered near 500 MHz, the novel feature of the recent findings including the present paper, is a less dramatic rotational immobilization of bound water compared to free water.

A similar picture results from our NMR measurements of the translational behavior of water. We find a decrease of the translational mobility of hemoglobin bound water by a factor of between three and five. Polnaszek and Bryant (1984) report a decrease of the translational mobility by a factor between five and 10 for hydration water of bovine serum albumin. Molecular dynamic simulations on lysozyme, bovine pancreatic trypsin inhibitor, and trypsin yield a decrease by a factor between two and five depending on the polarity of the nearby amino acid side chains (Brooks and Karplus, 1989; Teeter, 1991). Our result is in agreement with the cited results.

The translational diffusion coefficient D and the rotational correlation time τ for dielectric relaxation are related quantities. The interrelationship between D and τ may be derived easily using the Stokes-Einstein relation for translational diffusion and Debye's generalized Stokes-Einstein relation for rotational diffusion. If r denotes the radius of a spherical diffusing particle moving through a continuous, homogeneous fluid, this interrelation yields

$$\tau = 2r^2\kappa/(3D) \tag{25}$$

The solvent-dependent quantity κ is a measure of the ratio of the mean square intermolecular torque and the mean square intermolecular force (Kivelson et al., 1970), the range of possible values of κ is given by the inequality equation $0 < \kappa < 1$. The continuum property implies that the radius of the sphere is much larger than the radius of the solvent molecule. However, the Stokes-Einstein relations have been applied with much success in the analysis of the Brownian motion of particles with radii in the same order of magnitude as the radius of the solvent molecule. If we assume that the values of κ for bulk water and for water in the hydration shell differ only slightly, we conclude from Eq. 25 that an increase of τ should be accompanied by a related decrease of D. A comparison of our experimental results shows that rotational and translational processes in the hydration shell are indeed restricted by the interaction with the protein surface to a similar extent. In this connection, we have to consider that the kinetic parameters determined from the permittivity experiments are an average for the water molecules within a hydration shell of about 0.5-nm thickness, which results from

the hydration value b given in Table 1. In contrast to that, the relative translational motion of water molecules and the nitroxide contribute to the water proton magnetic relaxation within a distance of about 1 nm from the nitroxide (Polnaszek and Bryant, 1984). So less restricted motions of bulk water in the vicinity of the protein partly contribute to the relaxation process. Additionally we neglected a possible anisotropy of the translational diffusion mechanism. These features together with the limits of the NMR method discussed above justify the assumption that the diffusion parameters given in Table 2 are an upper limit. The translational as well as the rotational dynamics of water molecules involve a breaking and formation of hydrogen bonds. The entropies of activation of the rotational process are identical within their error limits for free water and hydration water (see Table 1). The small positive values of $\Delta S^{\neq}$ suggest that the water molecules reorient independently without substantial cooperative interactions. The comparable values of D_o for the translational motion (see Table 2) are also evidence that cooperative interactions for the translational motion in the hydration shell are similar to those in the bulk. Hence the dynamic processes are slowed in the hydration shell mainly due to the increase of the activation enthalpy. Compared to the values for free water, $\Delta H^{\neq} = 17$ kJ/mol, and for ice, $\Delta H^{\neq} = 55$ kJ/mol, the value of $\Delta H^{\neq} = 24$–30 kJ/mol for the hydration water may be evidence for a hydrogen-bonding structure in the bound water layer which is different from that of bulk water in the sense of an ice-lattice arrangement. From neutron diffraction experiments a localization of the hydration water protons to defined positions on the protein surface is deduced (Teeter, 1991). These positions are determined by intermolecular interaction of water molecules with specific surface sites of the protein. The results of the NMR and dielectric spectroscopy suggest that the water molecules jump between these specific sites with only slightly reduced mobility compared to that in the bulk.

CONCLUSION

Rotational correlation times and translational diffusion coefficients for water in the hydration shell of hemoglobin have been determined by means of dielectric permittivity measurements and nuclear magnetic resonance spectroscopy. The results are consistent with the presence of very mobile water on the protein surface. At physiological temperatures the rotational and translational correlation times of the main fraction of the so-called bound water are increased less than an order of magnitude compared to the respective values of bulk water. This observed decrease of the water mobility in the hydration shell compared to the bulk is mainly due to an enhanced activation enthalpy.

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