Fluctuations, exchange processes, and water diffusion in aqueous protein systems

A study of bovine serum albumin by diverse NMR techniques

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ABSTRACT Experimental frequency, concentration, and temperature dependences of the deuteron relaxation times T_1 and T_2 of D_2O solutions of bovine serum albumin are reported and theoretically described in a closed form without formal parameters. Crucial processes of the theoretical concept are material exchange, translational diffusion of water molecules on the rugged surfaces of proteins, and tumbling of the macromolecules. It is also concluded that, apart from averaging of the relaxation rates in the diverse deuteron phases, material exchange contributes to transverse relaxation by exchange modulation of the Larmor frequency. The rate limiting factor of macromolecular tumbling is determined by the free water content. In a certain analogy to the classical free-volume theory, a "free-water-volume theory" is presented. There are two characteristic water mass fractions indicating the saturation of the hydration shells ($c_s \approx 0.3$) and the onset of protein tumbling ($c_0 \approx 0.6$). The existence of the translational degrees of freedom of water molecules in the hydration shells has been verified by direct measurement of the diffusion coefficient using an NMR field-gradient technique. The concentration and temperature dependences show phenomena indicating a percolation transition of clusters of free water. The threshold water content was found to be $c_c^w \approx 0.43$.

1. INTRODUCTION

Nuclear magnetic relaxation of water nuclei in aqueous protein solutions is known to be governed by the interaction with the macromolecules (1–3). The same is true for generalized protein/water systems such as tissue (4–6). It is therefore of interest to understand the mechanisms taking place at the water/protein interface.

The study of the relaxation processes of water (including rapidly exchanging protein hydrogens) in particular is feasible by the aid of deuteron NMR in D_2O solutions (7, 8), because the results practically are not affected by cross-relaxation effects with (nonexchangeable) protein nuclei (6, 9). ^{17}O NMR is also of interest (7, 10) but is difficult to measure at low concentrations in the full frequency range of the field-cycling technique (11).

Numerous NMR relaxation studies of protein solutions characterizing the molecular dynamics in detail can be found in literature. With the exception of purely formal descriptions by correlation time distributions, for instance, there is, however, no theoretical concept describing in a closed way experimental data of *many* different measuring parameters in ranges as *wide* as possible. Furthermore, although the remarkably high mobility of water molecules in the hydration shells has been concluded with respect to rotational (e.g., 8, 12) and translational (e.g., 6, 8, 13, 14) diffusion, to our knowledge no direct measurements of diffusion of hydration water at very low water contents have been reported up to now.

In previous articles we published deuteron field-cycling relaxation data of D_2O solutions of bovine serum albumin (BSA) (8, 15). We now present an extended theory and additional data for the transverse relaxation time T_2 and the self-diffusion coefficient D. The study of the latter is suitable for the verification of the translational degrees of freedom of water molecules in the hydration shells. Thus the picture deduced from the relaxation studies can be supplemented. In particular, the model theory was confirmed by the observation of free-water/hydration water percolation phenomena (not to be confused with protonic conduction percolation observed by dielectric relaxation spectroscopy and reported in References 16 and 17).

The paper is organized as follows: section 2 provides a general definition and classification of the deuteron phases in protein/D_2O systems. Data characterizing the exchange properties are reviewed. The contributions of the theoretical spin-lattice and transverse relaxation rates of the phases and the general structure of the formulae for the total spin-lattice and transverse relaxation rates are presented in sections 3 and 4, respectively. In section 5, a fluctuation scheme is developed serving later for the interpretation of the experimental data and phenomena. One of the generally accepted motions in protein solutions

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is tumbling of the whole proteins. In section 6 we describe the "free-water-volume theory" taking into account the mutual hindrance of the proteins. In the experimental sections 7 and 8, deuteron relaxation in dependence of various measuring parameters is compared with the theory. The experimental proof that the crucial assumption of the fluctuation model, namely the translational degrees of freedom of hydration water, is justified, is presented in section 9, where direct diffusion experiments carried out with the NMR field-gradient technique are reported.

2. DEFINITION OF THE DEUTERON PHASES AND DISCUSSION OF THEIR EXCHANGE PROPERTIES

A well-established experimental finding in aqueous protein systems is the existence of "hydration water" at the interface (~30% by weight or, on average, a single monolayer of water molecules) which does not freeze at the regular water freezing temperature (18). This water phase rather remains fluid down to ~200 K.

With this definition of hydration water, deuterons in D_2O/protein systems may be classified in at least three phases according to the physical properties of the environment in which they are located. The phases and the corresponding fractions of the nuclear spin content are (*a*) free water (p_f), (*b*) hydration water (p_h), (*c*) exchange deuterons in the protein (p_p), where free water is defined complementarily to hydration water.

Exchange between the water phases can occur either by transfer of molecules or, depending on the pH value, of hydrogens. In the latter case the mean residence time in a water molecule was found to be in the order of 10^{-3} s at room temperature and pH 7 for protons (19, 20). The exponentiality of proton or deuteron spin-lattice relaxation curves observed in a frequency range down to a few kHz (2, 8) furthermore suggests that the mean residence times of water molecules are $<10^{-4}$–10^{-3} s.

Exchange of deuterons between water and amide groups in the interior of the protein can be neglected because it is very slow (21). These amide deuterons therefore do not contribute to the liquid-state signals which are of interest here. On the other hand, labile hydrogens which are directly accessible to the solvent can have high exchange rates even at neutral acidity. Typical exchange rate constants for OH, NH_2, and NH_3^+ groups at pH 7 and room temperature have been estimated to be $10^3\ s^{-1}$ (21). In the following, the exchange deuteron phase "*p*" will be identified with these solvent accessible, labile hydrogens. (Note that this restriction is not applicable for the discussion of proton relaxation because then one is confronted with additional spin diffusion and cross-relaxation phenomena [2, 6, 9].)

The deuteron fractions in the three phases obey

$$p_f + p_h + p_p = 1. \tag{1}$$

According to the above definition of the deuteron phases, exchange between the phases must be assumed to be beyond the limit of "slow exchange" (22) because the exchange rates are at least comparable to the relaxation rates intrinsic to the phases. Deuterons not fulfilling this limit are not considered here because their signals tend to be solid state signals which can be excluded in the measuring process.

3. CONTRIBUTIONS TO THE DEUTERON SPIN-LATTICE RELAXATION RATE

The following average formula applies for the total spin-lattice relaxation rate (compare references 22–24)

$$\frac{1}{T_1} = \frac{p_h}{T_1^h} f_1^h + \frac{p_f}{T_1^f} f_1^f + \frac{p_p}{T_1^p} f_1^p \tag{2}$$

with the rate fractions

$$f_1^j = \frac{\dfrac{1}{\tau_{ex}^j}}{\dfrac{1}{T_1^j} + \dfrac{1}{\tau_{ex}^j}} = \frac{T_1^j}{T_1^j + \tau_{ex}^j} \quad (j = h, f, p) \tag{3}$$

τ_{ex}^h, τ_{ex}^f, and τ_{ex}^p are the mean residence times in the three phases. The quantities f_1^h, f_1^f, and f_1^p represent the fractions of the maximum relaxation rates of the phases which effectively contribute to the total relaxation rate. The contributions of the phases are reduced by f_1^j because a part of the spins reaches the relaxed state before exchange, so that the volume occupied by them temporarily is "blocked" and does not participate in the equilibration process.

Based on the exchange rate data above mentioned, one must assume the "fast-exchange limits," $\tau_{ex}^h \ll T_1^h$, $\tau_{ex}^f \ll T_1^f$, and $\tau_{ex}^p \ll T_1^p$. Eq. 2 can then be approached by

$$\frac{1}{T_1} \approx \frac{p_h}{T_1^h} + \frac{p_f}{T_1^f} + \frac{p_p}{T_1^p}. \tag{4}$$

Apart from *averaging* of relaxation rates, exchange processes can also *modulate* spin interactions and provide an additional relaxation rate in this way. For deuterons, interactions which are exchange modulated can in principle be the quadrupole interaction with local electric field gradients, the Zeeman interaction with the local magnetic field and indirect coupling to other protein nuclei with spin.

All these interactions can provide secular and nonsecular terms. Spin-lattice relaxation is only affected by nonsecular terms, whereas transverse relaxation is dominated by unaveraged secular contributions (25). The Larmor frequencies relevant in this study are above 10^3 Hz, i.e., above the typical exchange rates. Even in the presence of nonsecular terms, therefore no exchange-modulation contribution to spin-lattice relaxation need be taken into account (compare reference 26).

4. CONTRIBUTIONS TO THE TRANSVERSE DEUTERON RELAXATION RATE

In contrast to spin-lattice relaxation, contributions from exchange modulation are relevant for transverse relaxation. Frequency shifts between the hydrogen phases have been discussed several times in the literature (27–30). The *Zeeman interaction* can be modulated by chemical shifts but also as a consequence of susceptibility changes at interfaces between materials with different densities (compare reference 31). With tensorial interactions there can be transverse components inducing nonsecular transitions. An analogous example is the "chemical shift anisotropy" mechanism (25). The influence of such anisotropy effects on the relaxation rates of hydrogens is known to be extremely weak particularly if the magnetic fields are low (32). Thus the Zeeman interaction can be considered to be scalar and merely a secular term contributes.

Indirectly spin-spin coupled protein deuterons normally interact with nuclei other than deuterons. Coupling constants for hydrogens bound to nitrogen, for instance, vary over a considerable range (33), but are strongly reduced due to the low gyromagnetic ratio of deuterons compared with protons. This interaction therefore can be neglected.

Quadrupole coupling is relevant if motional narrowing is incomplete as is often the case with proteins in more or less solid states (e.g., 34). If so, this interaction can provide the strongest effect in this context depending on the orientation of the field gradient with respect to the external field.

The influence of exchange modulation on Carr/Purcell/Meiboom/Gill (CPMG) echo trains was reported to be dependent on the external field (29, 30) suggesting that Zeeman modulation is more important than the field-independent couplings. Altogether we can define an effective difference of the Larmor frequencies $2\delta\omega_{i,j}$ between phases "i" and "j" (or a shift $\delta\omega_{i,j}$ from the midfrequency). In the literature, a series of treatments of the influence of exchange modulation on the transverse relaxation times of free-induction signals (35, 36), of two-pulse Hahn echoes (36, 37), and of CPMG echo trains has been reported (23, 24, 29, 36, 38, 39). According to these theories, additional transverse relaxation rates of the phase "i" due to secular exchange modulation processes with phase "j" are expected in the form

$$\frac{1}{T_2^{i,j}} = p_i p_j \tau_{ex}^i 4\delta\omega_{i,j}^2 g_{exp}. \tag{5}$$

g_{exp} is a numerical constant depending on experimental parameters like the CPMG pulse spacing. In the limit of long pulse spacings, its value tends towards 1. The fractions p_i and p_j of the nuclei a priori being in phases "*i*" and "*j*," respectively, have to be understood as time fractions that the nuclei spend in these phases. The exchange modulation process is most efficient if $p_i = p_j = 0.5$ is fulfilled.

Note that $\delta\omega_{i,j}$ may depend on the molecular orientation and the binding site location. Actually, even a continuous distribution $f(\delta\omega)$ of such frequency shifts may arise. In that case it is preferable to use the second moment M_2 of this distribution leading to the additional transverse relaxation rate (compare reference 37) of the phase "*i*" due to exchange to any other phase or site in the limit of long pulse spacings (i.e., $[g_{exp} \approx 1]$)

$$\frac{1}{T_2^i} = p_i(1 - p_i)4\tau_{ex}^i \int_0^\infty f(\delta\omega)\delta\omega^2 \, d\delta\omega$$

$$= p_i(1 - p_i)M_2\tau_{ex}^i. \tag{6}$$

The factor $(1 - p_i)$ is to represent the time fraction a nucleus spends outside of phase "i." The distribution function $f(\delta\omega)$ is expected to depend on the water content and, hence, the second moment will do so too.

The above treatment suggests a complicated pattern of exchange paths. It can, however, be expected that exchange modulation directly affects water nuclei mainly in the hydration shells. We will therefore restrict ourselves to the consideration of this phase alone in this particular respect. The second moment M_2 then characterizes the distribution of the Larmor frequency shifts outside of and relative to the hydration shell. The total transverse relaxation rate of the water is

$$\frac{1}{T_2^w} = \frac{p_f}{T_2^f} f_2^f + \frac{p_p}{T_2^p} f_2^p + \frac{p_h}{T_2^h} f_h^2 + (1 - p_h)p_h f_2^h M_2 \tau_{ex}^h. \tag{7}$$

The quantities

$$f_2^j = \frac{\dfrac{1}{\tau_{ex}^j}}{\dfrac{1}{T_2^j} + \dfrac{1}{\tau_{ex}^j}} = \frac{T_2^j}{T_2^j + \tau_{ex}^j} \quad (j = h, f, p) \tag{8}$$

are analogous to Eq. 3. Note that T_2 normally is much shorter than T_1. The rapid exchange case (compare Eq. 4) is therefore not necessarily fulfilled here.

5. FLUCTUATION SCHEME

The spin-lattice relaxation rates at the resonance frequency ω in the phases "k" are given by (25)

$$\frac{1}{T_1^k} = C_k[I_k(\omega) + 4I_k(2\omega)]. \quad (9)$$

C_k represents constants which depend on the interactions and on the type of the fluctuation. I_k is the intensity function of the fluctuations,

$$I_k(\omega) = \int_{-\infty}^{\infty} G_k(\tau)e^{-i\omega\tau}\,d\tau, \quad (10)$$

which is determined by the (reduced) autocorrelation function $G_k(\tau)$ decaying from the initial value 1 to 0 in the long-time limit.

The definition of the transverse relaxation time T_2 in the proper sense implies the "motional narrowing condition" which is experimentally justified at least for the water phases at water contents near or above the saturation of the hydration shells. The rate is then given by (25)

$$\frac{1}{T_2^k} = \frac{1}{2}C_k[3I_k(0) + 5I_k(\omega) + 2I_k(2\omega)]. \quad (11)$$

Intensity function for free water

The contribution from free water can be described by an exponential correlation function as it is valid for isotropic rotational diffusion (25). The correlation time in pure water at room temperature is $\tau_f \approx 3 \cdot 10^{-12}$ s (40) so that we may approximate for the experimentally accessible frequency range

$$I_f(\omega) = \frac{2\tau_f}{1 + \omega^2\tau_f^2} \approx 2\tau_f. \quad (12)$$

Intensity function for hydration water

In the hydration shells of biopolymers, the reorientation processes are of a different nature. The correlation function $G_h(t)$ for the nuclei of hydration water can be analyzed in terms of four contributions which virtually are independent of each other:

$$G_h(t) = G_\perp(t)G_l(t)G_r(t)G_i(t). \quad (13)$$

$G_\perp$ refers to the exchange with the surrounding free water, in which rotational diffusion is so fast that the correlation to the initial orientation is immediately lost. There may be a reentering process of water molecules so that a preferential orientation with respect to the surface is restored (41). Mechanisms of that kind will, however, implicitly be taken into account in the (effective) surface diffusion to be described below. The correlation time of

$$G_\perp(t) = \exp(-|t|/\tau_\perp) \quad (14)$$

is about equal to the mean residence time, τ_{ex}^h, of a nucleus in the hydration shell,

$$\tau_\perp \approx \tau_{ex}^h. \quad (15)$$

Within the hydration layers, a water molecule can be reoriented relative to the protein molecule in two ways. First it can perform restricted rotational diffusion about an axis perpendicular to the polar protein surface. The correlation function is expected to be

$$G_r(t) = a_1 \exp(-|t|/\tau_r) + a_2, \quad (16)$$

where $a_2 = 1 - a_1$ is the residual correlation in the long-time limit of this process.

On the other hand, water molecules are not attached permanently at definite adsorption sites. They rather underlie translational displacements along the protein surface with an effective diffusion coefficient D_l. Note that this mechanism does not exclude reentering of nuclei after temporary excursions to other phases. As the surface is rugged and because the water molecule adopt a preferential orientation relative to the polar groups, translations are connected with reorientations. We analyze the rugged surface by a spatial Fourier transformation in modes with wave numbers q. The upper and lower cut-off values, q_u and q_l, respectively, correspond to the dimensions of the system and the molecules involved in this process. In the range between these limits, all modes are assumed to be equally weighted.

The correlation function for the diffusion along a mode with the wave number q is written as an exponential function with the correlation time τ_q. During this correlation time a water molecule experiences, in the average, all different orientations connected with that mode. It is thus the time needed for a root mean square displacement equal to half of the wavelength of the mode or

$$\tau_q \approx 2.5/(D_l q^2). \quad (17)$$

The average correlation function for reorientations by surface diffusion is then

$$G_l(t) = \frac{d_1}{\Delta q}\int_{q_l}^{q_u} \exp(-|t|/\tau_q)\,dq + d_2 \quad (18)$$

with $\Delta q = q_u - q_l$ and $d_1 + d_2 = 1$. Here we have anticipated that the reorientations by this process are restricted so that a finite residual correlation d_2 remains in principle. d_2 is assumed to be the same for all modes and to be relatively small. For the experiments to be discussed, the limit $\omega \ll q_u^2 D_\parallel$ is valid, so that Eq. 18 can be approximated by

$$G_\parallel(t) \approx \frac{d_1}{\Delta q} \int_{q_l}^{\infty} \exp(-|t|/\tau_q)\, dq + d_2. \tag{19}$$

If structurally possible, tumbling of the whole macromolecular hydration complex must be regarded as a competitive mechanism. In the simplest case it is characterized by an exponential correlation function with the correlation time τ_t

$$G_t(t) = \exp(-|t|/\tau_t). \tag{20}$$

Combining the expressions for the partial correlation functions and approximating

$$\frac{1}{\tau_r} + \frac{1}{\tau_\perp} + \frac{1}{\tau_t} \approx \frac{1}{\tau_r} \tag{21}$$

leads to the total correlation function of nuclei in the hydration shell

$$\begin{aligned} G_h(t) = {} & (1 - a_2) \exp(-|t|/\tau_r) \\ & + \frac{a_2 d_1}{\Delta q} \int_{q_l}^{\infty} \exp\left\{-|t|\left(\tau_x^{-1} + \frac{2}{5} D_\parallel q^2\right)\right\} dq \\ & + d_2 \exp(-|t|/\tau_x) \end{aligned} \tag{22}$$

with

$$\frac{1}{\tau_x} = \frac{1}{\tau_\perp} + \frac{1}{\tau_t}. \tag{23}$$

The corresponding intensity function is

$$\begin{aligned} I_h(\omega) = {} & 2(1 - a_2)\tau_r \\ & + \frac{a_2 d_1}{\Delta q} \int_{q_l}^{\infty} \frac{2\left(\tau_x^{-1} + \frac{2}{5} D_\parallel q^2\right)^{-1}}{1 + \omega^2 \left(\tau_x^{-1} + \frac{2}{5} D_\parallel q^2\right)^{-2}} dq \\ & + a_2 d_2 \frac{2\tau_x}{1 + \omega^2 \tau_x^2}, \end{aligned} \tag{24}$$

where we have assumed $\omega\tau_r \ll 1$. The integral can be solved in the limits

$$\tau_x^{-1} \ll D_\parallel q^2 \tag{25}$$

and

$$\tau_x^{-1} \gg D_\parallel q^2, \tag{26}$$

where either the τ_x^{-1} or the $D_\parallel q^2$ term can be neglected. The cross-over between these limits is centered at

$$q_m = (\tfrac{2}{5}\tau_x D_\parallel)^{-1/2}. \tag{27}$$

The integral in Eq. 24 can be approximated by taking the limit Eq. 26 in the range $q_l \le q \le q_m$ and the limit Eq. 25 else. We define

$$\tau_\parallel = (\tfrac{2}{5} D_\parallel q_l^2)^{-1} \tag{28}$$

and

$$\frac{1}{\tau_m} = \frac{1}{\tau_\parallel} + \frac{1}{\tau_t} + \frac{1}{\tau_\perp}. \tag{29}$$

Then a closed formula for the whole parameter range can be established as an approach to Eq. 24

$$\begin{aligned} I_h(\omega) = {} & 2a_1\tau_r + k_h \left\{ \left(\frac{1}{\sqrt{\tau_m}} - \frac{1}{\sqrt{\tau_\parallel}} \right) \frac{2\tau_m}{1 + \omega^2 \tau_m^2} \right. \\ & + \frac{1}{\sqrt{2\omega}} \left[\frac{1}{2} \ln \frac{1 + \sqrt{2\omega\tau_m} + \omega\tau_m}{1 - \sqrt{2\omega\tau_m} + \omega\tau_m} + \pi \right. \\ & \left. \left. - \arctan\left(\sqrt{\frac{2}{\omega\tau_m}} - 1 \right) - \arctan\left(\sqrt{\frac{2}{\omega\tau_m}} + 1 \right) \right] \right\} \end{aligned} \tag{30}$$

with

$$k_h = \frac{a_2}{\Delta q \sqrt{\frac{2}{5} D_\parallel}}.$$

In the derivation of this expression the approximation $d_1 \approx 1$, i.e., $d_2 \approx 0$ has been adopted. The two limits which are of particular interest are $\tau_\parallel \ll \tau_t \ll \tau_\perp$ so that $\tau_m \approx \tau_\parallel$ and the opposite case, $\tau_t \ll \tau_\parallel \ll \tau_\perp$ leading to $\tau_m \approx \tau_t$.

Intensity function for exchange deuterons

The fluctuations of protein backbones can be described by the $\nu^{3/4}$ law reported previously in context with weakly hydrated proteins in the absence of molecular tumbling (6). It has been argued that side chain motions are not responsible for this behavior because they scarcely contribute in the frequency/temperature range of that study. One is rather confronted with fluctuations of the macromolecular backbone structure. The type of these fluctuations appears to be universal for proteins as far as one can judge from the available data. In reference 34, a model has been derived, which explains the $\nu^{3/4}$ law on the basis of multiple trapping, i.e., anomalous diffusion of dilating defects. We adopt this concept here without further discussion.

Tumbling of the macromolecules, be it restricted or not, must be regarded as a mechanism competitive to the intramolecular fluctuations. The correlation function therefore is a product of a term corresponding to the $\nu^{3/4}$ dependence and the correlation functions for tumbling. Let b_2 be the residual correlation of the intra-protein mechanisms in the long-time limit. $b_1 = 1 - b_2$ is defined analogously to a_1, a_2 in Eq. 16. Then

$$G_p(t) = (b_1 k_p t^{-0.25} + b_2) \exp(-|t|/\tau_t) \tag{31}$$

with k_p a constant. The intensity function of the protein nuclei is

$$I_p(\omega) = \left\{ 2 b_1 k_p \Gamma(0.75) \frac{\tau_t^{3/4}}{(1 + \omega^2 \tau_t^2)^{3/8}} \cdot \cos[0.75 \arctan(\omega \tau_t)] + b_2 \frac{2\tau_t}{1 + \omega^2 \tau_t^2} \right\}. \tag{32}$$

This equation is valid for frequencies not too high and temperatures not too low so that contributions from side chain motions can be neglected.

6. DEPENDENCE OF THE DEUTERON RELAXATION TIMES ON THE WATER CONTENT AND FREE-WATER-VOLUME MODEL FOR THE TUMBLING MOTION

The mass fraction of the water, c_w, enters into the above formalism via diverse parameters. These are especially the fractions p_f, p_h (p_p is neglected again) and the tumbling correlation time τ_t. The concentration dependences are subdivided by two characteristic water contents. Apart from the definition of the water mass fraction for saturated hydration shells, c_s, a critical water mass fraction, c_o, is introduced indicating when protein tumbling becomes comparably effective to surface diffusion.

In the following, we use the index i for the subscripts f, s, h, w, p, and o meaning "free," "saturation," "hydration," "water," "protein," and "critical," respectively. Thus we have the mass fractions c_i and the masses m_i which are related to each other by

$$c_i = \frac{m_i}{\sum_j m_j}; \quad m_i = \frac{c_i \left(\sum_j m_j - m_i \right)}{1 - c_i}; \quad \sum_j c_j = 1 \tag{33}$$

with $j = h, f$.

Free water can exist for two reasons, namely excess and activated water. If the mass fraction of the water obeys $c_w < c_s$, only the second origin can be relevant, whereas for $c_w > c_s$ both reasons contribute. For $c_w < c_s$, the fraction of nuclei in the free water is

$$p_f = \frac{m_f}{m_f + m_h} = \frac{1}{1 + \frac{m_h}{m_f}} = \frac{1}{1 + \exp(\Delta G/RT)}, \tag{34}$$

where ΔG is the difference of the molar Gibbs free energies in free and hydration water. For $c_w > c_s$, we have

$$p_f = \frac{m_w - m_s}{m_w} + \frac{m_s}{m_w} \frac{1}{1 + \exp(\Delta G/RT)} \tag{35}$$

or

$$p_f = \frac{1}{1 - c_s} \cdot \left[\frac{c_w - c_s}{c_w} + \frac{c_s}{c_w} (1 - c_w) \frac{1}{1 + \exp(\Delta G/RT)} \right]. \tag{36}$$

Tumbling of protein molecules is only possible if sufficient free water is available. One may adopt a kind of "free volume" theory previously developed for translational diffusion problems in condensed matter physics (42–44). "Free volume" is now interpreted as "volume of free water" and "translational diffusion" by "tumbling." One thus arrives at an analogous picture for molecular tumbling rates.

In the Cohen/Turnbull theory (42–44), one considers the redistribution of free volume in a sample. The original treatment referred to translational jumps requiring a minimum free volume v^* comparable to the volume of the molecule to be displaced. Now we are dealing with macromolecular tumbling. As the shape of globular protein molecules generally may be approached by oblate or prolate ellipsoids, a rotational jump is only possible if sufficient volume of free water is present in the neighbourhood. The probability, that a molecule finds an adjacent amount of free water exceeding the minimum volume v^* needed for rotational movements, is

$$P(v^*) = \exp(-\gamma^* v^*/v_f). \tag{37}$$

v_f is the volume of free water per protein molecule. γ^* takes into account the overlap of the free water volumes attributed to the protein molecules. Its value lies between $1/2$ and 1. v^* is related to the shape of the hydrated protein complex. The volume of the hydrated protein is $v_p + v_s$, where v_p and v_s are the volumes of the protein and its saturated hydration shell, respectively. The volume of an ellipsoid is $4/3\pi abc$, where a, b, and c are the major axes. Let c be the longest major axis. The minimum volume for (isotropic) rotational diffusion of the hydrated protein

molecule is then that of the circumscribing sphere with radius c. The ratio of the volumes of the circumscribing sphere and the ellipsoid is

$$r = \frac{c^2}{ab} \geq 1 \tag{38}$$

so that the excess volume of free water is

$$v^* = (r - 1)(v_p + v_s). \tag{39}$$

The correlation time of tumbling is then

$$\tau_t = \tau_t^0 \exp\{\gamma^*(r - 1)(v_p + v_s)/v_f\}. \tag{40}$$

τ_s^0 is the mean protein tumbling time for infinite dilution i.e., for the limit $v_f \rightarrow \infty$. It may be described by the Stokes/Einstein law

$$\tau_t^0 = \frac{3\eta_f(v_p + v_s)}{k_B T} \tag{41}$$

with the viscosity η_f of bulk water.

The volumes in the exponent in Eq. 41 are proportional to the masses (per protein molecule) and, hence, to the mass fractions, where the proportionality factors are approximately the same. Thus

$$\tau_t \approx \tau_t^0 \exp[\gamma^*(r - 1)(c_p + c_s)/c_f]. \tag{42}$$

In the absence of free water, i.e., for $c_f \rightarrow 0$, the tumbling time diverges as it must be. With

$$c_f \approx c_w - c_s; \quad c_p = 1 - c_w \tag{43}$$

the correlation time for tumbling becomes

$$\tau_t = \tau_t^0 \exp\left[\gamma^*\left(r - 1\right)\frac{1 - c_w + c_s}{c_w - c_s}\right] \quad \text{for} \quad c_w > c_s$$

$$= \infty \quad \text{else} \; . \tag{44}$$

The critical water mass fraction $c_w = c_0$ for the onset of protein tumbling is defined by the cross-over $\tau_t = \tau_\parallel$ or

$$\tau_\parallel = \tau_t^0 \exp\left[\gamma^*(r - 1)\frac{1 - c_0 + c_s}{c_0 - c_s}\right]. \tag{45}$$

Hence

$$c_0 = c_s + \frac{\tilde{\gamma}}{1 + \tilde{\gamma}} \tag{46}$$

with

$$\tilde{\gamma} = \frac{(r - 1)\gamma^*}{\ln(\tau_\parallel/\tau_t^0)} \; .$$

If $c_s \approx 0.3$ and $\tilde{\gamma} \approx 0.5$, we have $c_0 \approx 0.6$

7. EXPERIMENTAL

Bovine serum albumin was purchased from Behring-Werke (Marburg/Lahn, FRG). The electrophoretic purity was specified as 100%. The material was lyophilized before the sample preparation so that a definite initial state was given (water content roughly 1.6% by weight). Samples with water contents <30% were prepared by exposing the lyophilized protein to a humid atmosphere until the desired concentration was reached. Above 30%, three times distilled water was added by the aid of an Eppendorf pipette. All water contents were determined by weighing and are given as the percentage of water (exclusive the content in the lyophilized state) in the total sample.

The experiments concerning the deuteron T_1 dispersion in D_2O solutions of bovine serum albumin are described in reference 8. The home-made apparatus has been described elsewhere (11). The T_2 data were measured with the conventional Hahn spin-echo method and compared with free-induction decays using a Bruker Instruments, Inc. (Billerica, MA) MSL 300 spectrometer with a magnetic flux density of 7 T. All relaxation curves could be fitted reasonably well with monoexponential decay functions over at least one decade. The deuteron spectra above 10% D_2O consequently were Lorentzian and no quadrupole splitting was perceptible under such circumstances. (This is in contrast to deuteron exchanged but dry proteins, of course [6, 34].) The principle of the diffusion measurements was the NMR field-gradient method in the stimulated echo version (45). Instead of rectangular gradient pulse shapes, a shifted cosine function as described in references 46 and 47 was used. A description of the home-made apparatus and the evaluation procedure is published elsewhere (48).

8. COMPARISON WITH DEUTERON RELAXATION EXPERIMENTS

The above formalism provides the means for the discussion of a series of NMR experiments. We begin with the consideration of the deuteron T_1 dispersion.

Deuteron T_1 dispersion

Figs. 1, *a* and *b*, show exemplary sets of deuteron T_1 dispersion data. The dispersion step begins at frequencies greater than a certain "inflection frequency"

$$\nu_i = \frac{1}{2\pi\tau_m} \; . \tag{47}$$

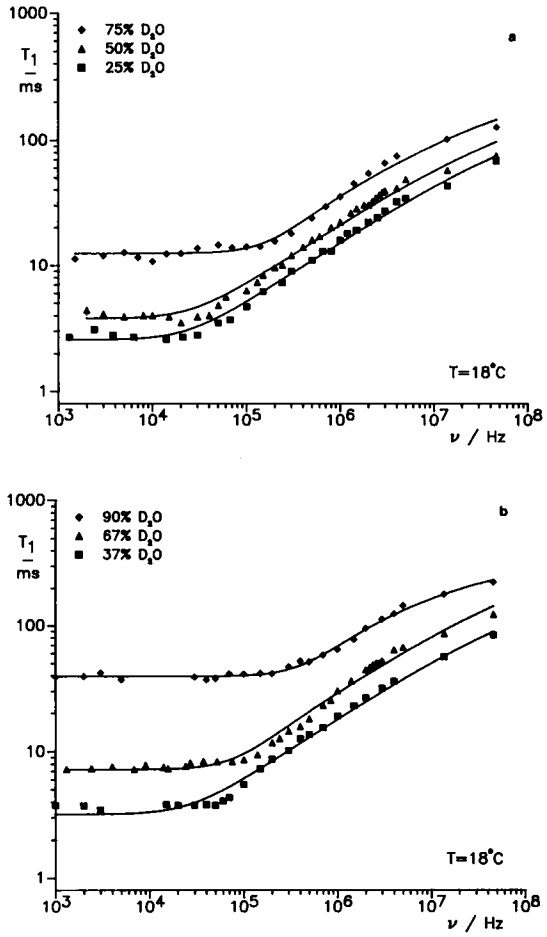

FIGURE 1 Frequency dependence of the deuteron spin-lattice relaxation time in D_2O solutions of bovine serum albumin (data from reference 8). The water contents are given in percent by weight. The solid lines were calculated as described in the text. As concentration dependent parameters, the quantities $\kappa_1 = C_h k_h$ and $\kappa_2 = 10(p_f/T_1^f) + (1 - p_f)C_h(1 - a_2)\tau_r$ were fitted to the data. The values for $c_w = 0.25$, 0.37, 0.50, 0.67, 0.75, 0.90 are $\kappa_1 \cdot 10^{-4}\ s^{3/2} = 1.82, 1.93, 2.75, 4.01, 4.51, 5.96$, and $\kappa_2 \cdot s = 4.26, 3.39, 3.80, 2.25, 3.31, 2.75$, respectively. Concentration independent parameters are $\tau_t^0 = 5 \cdot 10^{-8}$ s and $\tau_\parallel = 4 \cdot 10^{-6}$ s. $\tau_\perp$ turned out to be $\gg \tau_\parallel$ and has therefore no influence.

The solid lines in Figs. 1, *a* and *b*, represent the model theory. Here we have taken advantage of the inequalities $\tau_\perp \gg \tau_t$, $\tau_\parallel \gg \tau_f$, τ_r, which are justified by the typical orders of magnitude $\tau_\perp \approx 10^{-3}$ s and $\tau_f \approx 10^{-12}$ s. Furthermore the influence of the protein deuterons is not considered (it will, however, be taken into account in context with the exchange-modulation effect on T_2). There is an experimental justification for the neglect of p_p: even at low water concentrations when protein tumbling does not take place, a low-frequency plateau of the T_1 dispersion appears instead of the $\nu^{3/4}$ law found for the T_1 dispersion of protein hydrogens (6, 34).

Concentration dependences

Figs. 2, *a–c*, show the dependences on the mass fraction of water c_w of the transverse deuteron relaxation time T_2 and of the inflection frequency $\nu_i = (2\pi\tau_m)^{-1}$. The linewidth of the water component has also been evaluated for comparison. The critical mass fraction $c_0 \approx 0.6$ manifests itself as the cross-over from the case

$$c_w < c_0; \quad \tau_t \gg \tau_\parallel; \quad \nu_i = \nu_i^{\min} = \frac{1}{2\pi\tau_\parallel} \tag{48}$$

to

$$c_w > c_0; \quad \tau_t \ll \tau_\parallel; \quad \nu_i = \frac{1}{2\pi\tau_t} \tag{49}$$

A slight deviation between Hahn and linewidth data can be stated (Fig. 2 *b*). With the high-resolution instrument used, it cannot be explained by magnet inhomogeneities. Rather a partial compensation of the exchange modulation effect due to the 180° refocusing pulse of the echo sequence has to be assumed.

The solid line in Fig. 2 *c* has been calculated by the aid of the above formalism. p_p has been neglected and the exchange modulation term in Eq. 7 has not been taken into account. f_2^f has been approximated by 1. A constant value of $\kappa_1 \equiv k_h C_h$ has been assumed. The theoretical curve approaches the data but obviously does not describe them quantitatively at low water contents. At $c_w = 0.3$ the theoretical T_2 values exceed the experimental data by 100%. The discrepancy would tend to be even more severe if the concentration dependence of κ_1 as fitted to the T_1 dispersion data (see legend of Fig. 1) would have been taken into account. The conclusion is that the exchange-modulation term in Eq. 7 contributes correspondingly strongly. Unfortunately there is no theory for the concentration dependence of the second moment M_2 of the distribution function $f(\delta\omega)$ available at present so that no direct fit could be tried.

The cross-over at $c_s \approx 0.3$ from $c_w < c_s$, that is in the absence of free water, to $c_w > c_s$, where the increasing free water content decreases the average relaxation rates is also visible in Fig. 2 *b*. Experimentally this phenomenon becomes more pronounced at temperatures where the free water is frozen, but the hydration water is still liquid (Fig. 3). A strong concentration dependence appears for $c_w < c_s \approx 0.3$ which is attributed to a reduction of the effective water diffusion coefficient $D_\parallel$ and to an increase of M_2.

The fits to the T_1 dispersion data suggest a concentration dependence of

$$\kappa_1 = C_h \frac{a_2}{0.4\Delta q D_\parallel} \tag{50}$$

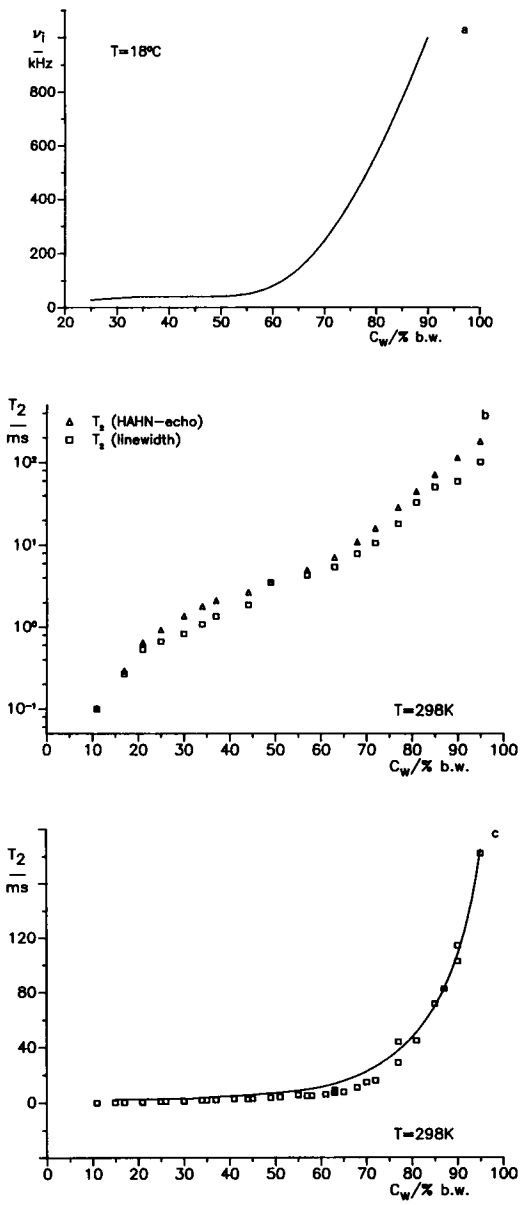

FIGURE 2 Dependences on the water mass fraction: (*a*) $\nu_i = 1/(2\pi\tau_m)$ corresponding to the theoretical lines of Figs. 1, *a* and *b*. (*b*) Deuteron T_2 of BSA/D_2O solutions evaluated from Hahn echo decays and linewidths. The data have been recorded with a high-resolution instrument at 7 T. The breaks at the two characteristic water mass fractions, $c_s \approx 0.3$ and $c_0 \approx 0.6$ are clearly visible. (*c*) Tentative comparison of the deuteron Hahn T_2 data with a model curve. The parameters $\tau_t^0 = 5 \cdot 10^{-8}$ s, $\tau_\parallel = 4 \cdot 10^{-6}$ s, $\tau_\perp = 4.5 \cdot 10^{-4}$ s are the same as with T_1. $\kappa_l = 5.4 \cdot 10^4\ s^{-3/2}$ was kept constant in the whole range of water contents. In contrast to exchange averaging, the contribution by exchange modulation (Eq. 6) has not been taken into account (see Discussion). The curve part for $c_w < c_s$ has been calculated by the aid of an empirical expression (15).

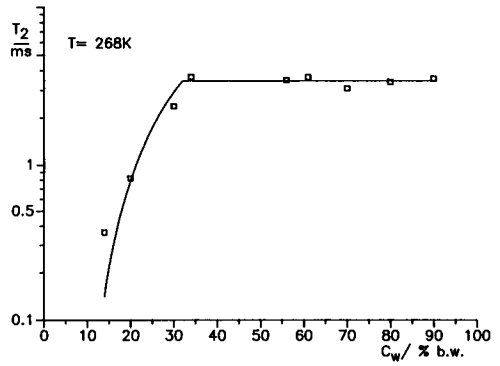

FIGURE 3 Transverse proton relaxation time of hydration water vs. the water mass fraction of frozen H_2O solutions of BSA. The temperature was chosen so that the free water was frozen and did not contribute to the Hahn echo signal in contrast to hydration water. Below the saturation concentration $c_s \approx 0.3$ a strong decrease of the relaxation times appears indicating a reduction of the mobility of the hydration water molecules.

(see legend of Fig. 1). The increase of κ_l by a factor of 3 while the water content rises from 25 to 90% is not yet fully understood. It very likely indicates the variation of q_l and hence of Δq with the water content: the hydration shells overlap more and more with higher protein concentrations and the protein molecules tend to align with respect to each other because of the mutual interaction (13). The consequence is that "intermolecular" and hence longer mode wavelengths become more and more relevant.

Temperature dependences of the transverse deuteron relaxation time

Regarding signals of liquid water only (Fig. 4), we have $p_f = 0$ and $p_h = 1$ for temperatures below 0°C (p_p is neglected again). Above the freezing point the normal fractions as given by Eqs. 35 and 36 are relevant. Explicit temperature dependences are obvious with the p_f expressions and τ_t^0. Other parameters are implicitly temperature dependent. We consider in particular η_f, $D_\parallel^{-1}$, τ_f, and $\tau_\perp$. As all of these quantities refer to water, we may assume a common Arrhenius law with a single apparent activation energy E_a. Thus

$$\eta_f, \tau_f, \tau_\perp, D_\parallel^{-1} \propto \exp(E_a/k_B T). \tag{51}$$

The lines in Fig. 4 have been calculated on the basis of these assumptions using the parameters of the T_1 dispersion data, and the above formalism omitting again the unknown exchange-modulation term of Eq. 7. The reduction of T_2 at the highest temperatures is due to denaturation (29). It is therefore not implied in the model description. The theoretical values tend to be larger than

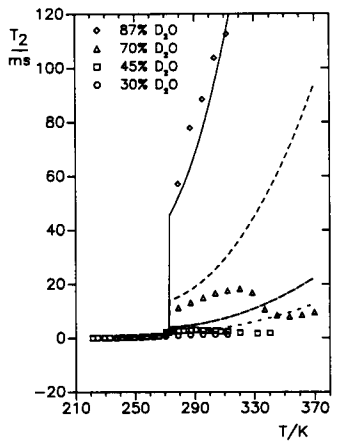

FIGURE 4 Temperature dependences of the transverse deuteron relaxation times of D_2O solutions of BSA. The sudden change at the freezing point of the free water is due to the cross-over to $p_f = 0$ and $\tau_t \rightarrow \infty$ in the frozen state (the ice signal was not recorded [49]). The reduction at high temperatures is due to denaturation of the protein (29). The lines were calculated by the aid of Arrhenius laws for all time constants and diffusion coefficients. The apparent activation energy was assumed to be 20 kJ/mol. All other parameter values were the same as or equivalent to those used in Fig. 2 *c*. The influence of denaturation has not been taken into account.

the experimental data (for the native state). This tendency increases with lower water contents. Deviations of ~100% were found with the 30% D_2O sample, for instance. We attribute this discrepancy to the exchange-modulation effect again as discussed above in context with the concentration dependence.

The jumplike transition of the T_2 data at the freezing point of free water permits the direct evaluation of p_f. The data above and below the freezing point have been extrapolated to the transition temperature. Fig. 5 shows the evaluated p_f values in comparison with the theoretical curve (Eq. 36).

9. VERIFICATION OF THE TRANSLATIONAL DEGREES OF FREEDOM OF HYDRATION WATER BY DIRECT DIFFUSION MEASUREMENTS

Temperature dependence of the water diffusion coefficient

Fig. 6 shows the temperature dependence of the water diffusion coefficient of two different BSA solutions. In the 41% sample, diffusion coefficients were measured even at −10°C. No abrupt change was visible at the freezing temperature albeit the water content was above the saturation concentration of hydration water (~30%). This is in contrast to the 90% sample which showed a strong reduction of the diffusion coefficient when free water was frozen. A more detailed study concerning diffusion in the frozen state and the discussion of the anomaly of the diffusion found under such circumstances will be published elsewhere.

The conclusion is that a percolation threshold is located between these two water contents: in the frozen samples, the liquid hydration shells surrounding the globular BSA molecules form restricted clusters embedded in free-water ice. Above the freezing temperature, diffusion in free water can "short-cut" the diffusion pathways in the hydration shells provided that it forms infinite percolation clusters. This appears to be fulfilled for the 90% sample in contrast to the 41% sample.

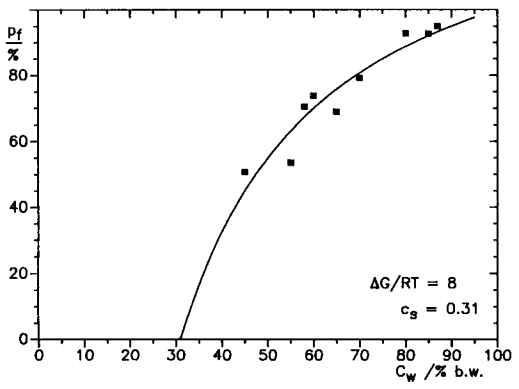

FIGURE 5 Dependence of p_f on the water mass fraction c_w. The data have been evaluated from the T_2 jumps at the freezing point. The curve has been calculated by the aid of Eq. 36.

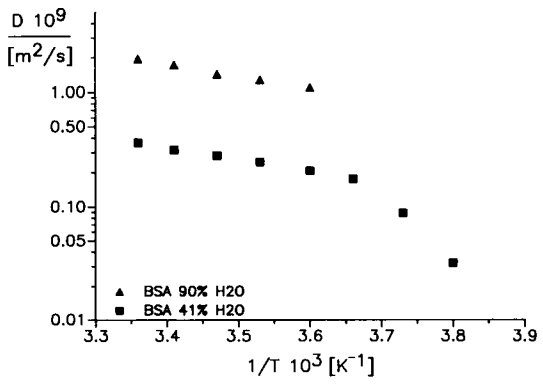

FIGURE 6 Temperature dependence of the water diffusion coefficient in BSA solutions with water contents of 41% and 90%. The diffusion time was 10 ms.

Concentration dependence of the water diffusion coefficient

Fig. 7 finally shows the concentration dependences of the water diffusion coefficient in BSA solutions at different temperatures. A cross-over is visible at $c_w = c_c^w \approx 43\%$. Note that this value lies above c_s. This phenomenon again must be considered to be indicative for the percolation transition of the free-water compartments. Diffusion becomes slower as soon as the percolation clusters of the free water become finite. One then has only "islands" of free water and the measurable displacements take place mainly in hydration water. Below $c_w = c_s = 30\%$ the free-water islands finally disappear completely. In the following we present a model theory predicting this "free-water percolation" behavior of the data in Fig. 7 (fitted curves).

Interpretation of the concentration dependence of the water diffusion coefficient

The mean square displacements of a water molecule are composed of contributions of both water phases. In the frame of this semi-quantitative interpretation, we may assume that the diffusion process behaves normally, i.e., that a linear time dependence of the mean square displacement is applicable:

$$\langle r^2 \rangle = 6Dt. \tag{52}$$

(Deviations from this law will be reported and discussed elsewhere.) Linearity means that we can superimpose

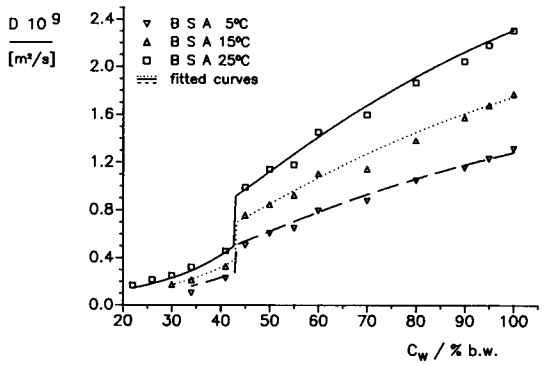

FIGURE 7 Concentration dependence of the water diffusion coefficient in BSA solutions at various temperatures. "Free-water percolation transitions" are visible. The fitted curves have been calculated as described in the text. The parameters are $A_1 = 0.125$; $A_2 = 4.5$; $A_3 = 0.714$; $v_p = 0.75$ cm³/g; $\alpha = 1.53$; $c_s = 0.27$; $c_c^w = 0.43$; $\Delta G/RT = 8$. The measured values of the diffusion coefficients in bulk water, D_0, are given by the last data points on the right. The diffusion time was 10 ms in all cases.

mean square displacements in different compartments. Let us designate the diffusion coefficient in free water and hydration water by D_f and D_h, respectively. The effective diffusion coefficient which is measured in the experiment is then composed according to

$$D = p_f D_f + p_h D_h, \tag{53}$$

where p_f and $p_h = 1 - p_f$ are the time fractions a water molecule diffuses in the free and the hydration water, respectively. p_f is given by Eq. 36.

The free-water diffusion coefficient D_f is reduced compared with the bulk water diffusion coefficient D_0 by the obstruction effect of the macromolecules (50, 51). At low protein concentrations we have (51)

$$D_f = D_0 \frac{1 - \alpha\Phi}{1 - \Phi} \tag{54}$$

with the volume fraction of the hydrated proteins

$$\Phi = \frac{c_p v_p + c_h v_h}{c_p v_p + c_h v_h + c_f v_f}. \tag{55}$$

v_p, v_h, v_f are the specific volumes of proteins, hydration water, and free water, respectively. α is a numerical parameter taking into account the shape of the protein with respect to the obstruction effect. (We are aware that at higher protein concentrations there might be deviations from the behavior represented by the above equation [50, 51]).

Above the saturation concentration of the hydration shells,

$$c_w = c_f + c_h = 1 - c_p \geq c_s \approx 0.3, \tag{56}$$

we may use

$$c_h = c_p \frac{c_s}{1 - c_s}. \tag{57}$$

The diffusion coefficient within the hydration shells depends on the saturation degree. For $c_w < c_s$, water molecules more and more become trapped on theeasurface and/or the diffusion paths along the surface become restricted. We take this into account by using the empirical relation

$$D_h = D_0 \frac{A_1}{1 - c_w} \exp\left[-A_2(1 - c_w - c_c^w)\right] \quad (c_w \leq c_c^w). \tag{58}$$

A_1, A_2 are numerical constants, c_c^w is the threshold water concentration of the free-water percolation process. Above c_s, the diffusion coefficient in the hydration shells is assumed to be constant

$$D_h = A_3 D_0. \tag{59}$$

For fitting purposes, the denominator of Eq. 55 is approximated by 1 cm^3/g. We further assume that $v_f \approx v_h \approx 1\ cm^3/g$. Eq. 55 thus becomes together with Eq. 57

$$\Phi \approx c_p\left(\frac{v_p}{[1\ cm^3/g]} + \frac{c_s}{1 - c_s}\right). \quad (60)$$

For $c_w < c_c^w$, molecules in the free-water phase cannot contribute to displacements being large enough for the measurement. Therefore one can set $p_f = 0$ in this case. The lines in Fig. 7 have been calculated by the aid of the above formalism. The good coincidence with the experimental data supports the assumption of a free-water percolation mechanism.

10. DISCUSSION

A closed model theory of deuteron relaxation in D_2O/protein systems has been presented. We have considered the frequency, concentration, and temperature dependences of the relaxation times and the parameters influencing them. Exchange modulation appears to have a nonnegligible influence on T_2. Moreover we have shown by diffusion experiments that hydration water in bovine serum albumin solutions has translational degrees of freedom. The high mobility is maintained even below the freezing point of free water. It has also been demonstrated by water diffusion measurements in myoglobin single crystals (48).

It is known that water in protein systems shows orientational correlation times up to an order of microseconds. This is longer than in bulk water by six orders of magnitude. A frequent conclusion near at hand therefore was that hydration water is irrotationally bound at the protein surface. This notion is ruled out by the present study: water molecules appear to remain "locked" within the hydration shells for longer periods, but nevertheless are able to diffuse along the surface of the protein.

There might be contributions to "surface" diffusion from spins transiently leaving the hydration water phase to free water (41, 52). The corresponding water molecules would restore their orientation relative to the protein surface depending on the reentering location. The spins therefore would continue to participate in the process of correlation decay of hydration water. A simple computer simulation of the reentering probability from the free water phase predicts, however, that the reentering probability should be small so that the majority of the hydration water molecules should stay in the hydration shell during the whole residence period of $\sim 10^{-6}$ s. An appreciable contribution from reentering water molecules would also be expected to reveal itself by a strong concentration dependence of $\tau_\parallel$ above c_s that has not been observed. Statements concerning reentering in a time scale beyond 10^{-6} s, on the other hand, are not possible on the basis of the correlation times, of course.

The nature of the mechanism locking water molecules to the hydration shells for times exceeding 10^{-6} s is not yet quite clear. Very likely it is more a thermodynamical problem of the different and incompatible microstructures in free and hydration water, so that rather high binding energies apparently appear.

Interestingly it was found that hydration water of lipid bilayers shows quite analogous phenomena indicating a certain universality of the behavior of hydrated surfaces. The deuteron spectrum of D_2O/lipid systems is strongly motionally narrowed, but there is still a residual quadrupole splitting (53–55). This indicates a preferential orientation of the water molecules with respect to the bilayer surface. The splitting depends on the water content but is still present when the hydration shells are saturated and a bulk water phase exists. One can again conclude that the mean residence time of a deuteron in the hydration shells exceeds the time scale of deuteron spectra, i.e., at least 10^{-6} s. Within this time scale, surface diffusion takes place. It causes no major narrowing of the quadrupole splitting, if the surfaces are virtually planar as it is the case in the so-called $L_{\beta'}$ and L_α phases. In the intermediate "ripple phase" P'_β of dipalmitoylphosphatidylcholine bilayers, however, the quadrupole splitting disappears (55) as a consequence of fast diffusion over the ripple period of ~150 Å.

The above fluctuation scheme for deuterons in protein/D_2O systems explains most of the experimentally observed phenomena in a physical way without formal parameters. Nevertheless, further work is needed with respect to the exchange modulation effect: the deuteron T_2 values of the BSA/D_2O systems were found to be lower than expected from the set of parameters fitted to the T_1 dispersion alone. the conclusion is that exchange modulation processes provide an additional contribution to transverse relaxation but are ineffective for the T_1 dispersion at least at frequencies well above the exchange rates (compare reference 26).

The percolation cross-over effects observed with water diffusion directly demonstrate the existence of "free water" and "hydration water" compartments with different diffusion coefficients. The percolating cluster thus either refers to free water or to hydration water compartments depending on the kind of experiment. These phenomena should not be confused with those observed with proton conduction along threads of H-bonded H_2O molecules (16, 17): the threshold water concentration reported in this respect is at least three times lower than that for the water diffusion percolation found in this study.

Diffusion on percolating clusters is expected to behave anomalously (56–58). In principle such behavior should be observable with the NMR method (59, 60). It reveals itself by a dependence of the evaluated diffusion coefficient on the diffusion time. This has indeed been found in a preliminary investigation, which will be presented and discussed elsewhere in more detail. Note that all of the above diffusion coefficients were measured with a fixed diffusion time of 10 ms guaranteeing that their relative values are comparable.

The high mobility of the hydration water is also demonstrated by the deuteron lineshape at low water contents. For $c_w = 20\%$ and at room temperature, we have found an Lorentzian-like line with a width of 550 Hz at half height. This is in contrast to the powder patterns observed with exchange deuterons in proteins (14, 34). With very extended protein systems like collagen fibers, on the other hand, a clear dipolar or quadrupolar splitting of the water line has been observed (61). This observation fits to the expectations of the surface diffusion model: surface translations are then less efficient in averaging out secular broadening.

^{17}O data show that hydration water is less mobile than free water, and that its motions are anisotropic (13). This confirms first that $\tau_\perp \gg \tau_t$ and second that it is mainly the motions of water molecules rather than fluctuations of exchange hydrogens that determine the T_1 relaxation behavior. The T_2 data of this study, the different T_1/T_2 ratios for hydrogens (^1H or ^2H) and ^{17}O (62), and CPMG experiments (29, 30) strongly suggest the influence of exchange modulation of the Larmor frequency.

The diffusion coefficients measured in the concentration range below the percolation threshold c_c^w (Fig. 7) may be compared with the correlation time for surface diffusion, $\tau_\parallel$ (Eq. 28). The room temperature values of D and $\tau_\parallel$ are $\sim 2 \cdot 10^{-10}$ m^2/s and $4 \cdot 10^{-6}$ s, respectively. (By the way, the D value agrees very well with the data of Polnaszek and Bryant [63] measured with a completely different technique.) Tentatively equating $D_\parallel$ with the directly measured quantity D leads to a longest mode wavelength of $\sim 10^{-7}$ m. For the present problem, only half of the wavelength is relevant or 500 Å. The circumference of a BSA molecule is ~300 Å for comparison. The value of the longest mode wavelength appears to be reasonable if one regards that a water molecule can diffuse during $\tau_\parallel$ several times around a protein molecule and can be displaced in the overlapping hydration shells along several adjacent protein molecules until the orientation correlation has decayed.

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