DEUTERON FIELD-CYCLING RELAXATION SPECTROSCOPY AND TRANSLATIONAL WATER DIFFUSION IN PROTEIN HYDRATION SHELLS

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ABSTRACT The deuterated hydration shells of bovine serum (BSA) albumin, and purple membrane sheets have been studied by the aid of deuteron field-cycling relaxation spectroscopy. The deuteron Larmor frequency range was 10^3 to 10^8 Hz. The temperature and the water content has been varied. The data distinguish translational diffusion on the protein surface from macromolecular tumbling or exchange with free water. A theory well describing all dependences has been developed on this basis. All parameters have successfully been tested concerning consistency with other sources of information. The concept is considered as a major relaxation scheme determining, apart from cross-relaxation effects, the water proton relaxation in tissue.

INTRODUCTION

Spin-lattice relaxation of water nuclei in cells and tissue is remarkably sensitive to the type and state of the system (1,2). It turned out that it is the macromolecular constituents which act as relaxation sinks via exchange mechanisms (3,4). Aqueous protein solutions have extensively been studied by proton spin-lattice relaxation (e.g., 5–9). It has been shown that both relaxation mechanisms within the hydration shells and as well as within the protein molecules are relevant. The latter contribution enters through cross-relaxation processes and manifests itself for instance through the characteristic $^{14}N^1H$- and $^2H^1H$-quadrupole dips (4).

Here, our interest is devoted particularly to the contribution from the hydration shells. Using deuteron nuclear magnetic resonance ensures both the specific location where the relaxation behavior is to be studied and the justification to neglect any internuclear interactions. The dominance of quadrupolar coupling of the deuterons to electric field-gradients as a perturbation mediating deuteron relaxation, permits us to separate the intrahydration shell mechanisms from cross-relaxation with nuclei at the protein surface. As a consequence, no cross-relaxation quadrupole dips (4) can be observed with deuterons.

EXPERIMENTAL

The field-cycling relaxation spectrometer and the technique has been described elsewhere (10). The data for deuteron frequencies above 10^7 Hz have been recorded with conventional pulse spectrometers. Bovine serum albumin (BSA); electrophoretic purity 100%) has been purchased from Behring-Werke, Marburg/Lahn, FRG. The purple membrane sheets (PM) have been prepared in this lab (11). The samples were lyophilized for 24–36 h, dissolved in D_2O and concentrated to the desired water contents by partial lyophilization. All concentrations are given in percent D_2O by weight. 0% water is defined by the state reached after the drying procedure described above.

Due to the limited bandwidth of the spectrometer and the short relaxation times, reliable measurements were rather hard below 25% water concentration. For the same reasons, signals from exchange deuterons of the proteins did not contribute to the measured relaxation rates with the exception of a small amount of atoms exchanging sufficiently fast compared with T_1. As a consequence, all relaxation curves could be described by single exponentials.

THEORETICAL CONCEPT

A two-site, rapid exchange model referring to free water and hydration water is assumed. The hydration shell is defined as the space region around the protein molecule where a preferential orientation of the water molecules due to the interactions with the polar groups of the protein exists. Protein hydrogen atoms potentially exchanging rapidly enough with those of water are associated with the hydration shell. Within the hydration shells anisotropic translational and rotational degrees of freedom are taken into account. Diffusion is modeled by a continuous process rather than by a hopping mechanism. The hydration water molecules are assumed to "see" a rugged protein surface formed by the outer polar groups. The translational degrees of freedom in the hydration shells are considered to be quasi two-dimensional as long as the exchange with the free water is not the rate-limiting process.

The consequence of this ansatz is that translations along the surface are correlated with reorientations of the water molecules. The curvature and the finite size of the globular protein structures thus can provide the long time cut off of reorientations.

The rugged surface can be analyzed in spatial Fourier modes with wave numbers $q = 2\pi\lambda^{-1}$ corresponding to a wavelength λ. The limits are given by the dimensions of the

water (upper limit q_u) and the protein (lower limit q_ℓ). Between these limits the spectral mode density is considered to be "white."

Let $D_\parallel$ be the translational diffusion coefficient along the protein surface. The orientation correlation function for water molecules is then composed of mode contributions (compare 12)

$$G_q(t) = \exp(-|t|/\tau_q) \tag{1}$$

with

$$\tau_q = (D_\parallel q^2)^{-1}.$$

The average orientation correlation function due to translations parallel to the protein surface is

$$G_\parallel(t) = \frac{1}{\Delta q}\int_{q_\ell}^{q_u} G_q(t)\, dq \tag{2}$$

with

$$\Delta q = q_u - q_\ell.$$

As a second process possibly contributing to the decay of the orientation correlation, we consider the exchange with the free water

$$G_\perp(t) = \exp(-|t|/\tau_\perp) \tag{3}$$

with $\tau_\perp = d^2/2D_\perp$. ($D_\perp$, diffusion coefficient within the hydration shell perpendicular to the protein surface; d, effective thickness of the hydration shell). $\tau_\perp$ is the mean time a water molecule needs to diffuse from the hydration to the free water phase, i.e., by translations perpendicular to the protein surface. Once a water molecule has reached the free-water phase, it will be reoriented in a very short time (10^{-12}–10^{-11} s) so that the diffusional exchange can be taken as a rate-limiting process.

Rotational diffusion about axes perpendicular to the protein surface provides a third intra-hydration shell contribution possibly influencing the relaxation behavior. We assume a partial correlation function

$$G_r(t) = a_1 \exp(-|t|/\tau_r) + a_2 \tag{4}$$

with $a_1 + a_2 = 1$. a_2 is the residual correlation characterizing the anisotropy of this process.

In principle the rotational diffusion about axes perpendicular to the protein surface could be correlated to the translational diffusion steps parallel to the protein surface. The assumption that Eqs. 2 and 4 are independent from each other would then not be justified. On the other hand, many translational diffusion steps are required for the long wavelengths modes until the reorientation by surface diffusion becomes perceptible. Thus, even if the elementary steps of the rotational and translational degrees of freedom are correlated, the mean square displacements parallel to the protein surface effectively are not. This may also be rationalized by considering the different timescales to be assumed: $\tau_q \gg \tau_r$.

Finally, the whole protein/water complex can tumble in dilute solutions leading to a correlation function

$$G_t(t) = \exp(-|t|/\tau_t). \tag{5}$$

(For simplicity we assume that the tumbling is isotropic.)

As all motions can be considered to be stochastically independent, we have the total orientation correlation function

$$G(t) = G_\parallel(t)\, G_\perp(t)\, G_r(t)\, G_t(t). \tag{6}$$

This function can be simplified by taking the limits $\tau_r \ll \tau_q$, τ_t as guaranteed. Then

$$G(t) \approx a_1 \exp(-|t|/\tau_r) + a_2\, G_\parallel(t)\, G_\perp(t)\, G_t(t). \tag{7}$$

In the limit $t \gg \tau_r$, which is expected to be valid at least for the lower frequencies of this study, we can use as a further approximation

$$G(t \gg \tau_r) \approx a_2\, G_\parallel(t)\, G_\perp(t)\, G_t(t). \tag{8}$$

The corresponding intensity function is given by the Fourier transform expressed as twice the real part of the Laplace transform for the imaginary variable $s = i\omega$

$$I(\omega) = 2\,\mathrm{Re}\{\mathcal{L}\,[G(t)]_{s=i\omega}\}. \tag{9}$$

Introducing $\tilde{s} = i\omega + \tau_x^0$, where

$$\tau_x^{-1} = \tau_\perp^{-1} + \tau_t^{-1}, \tag{10}$$

we obtain

$$I(\omega) = 2a_2\,\mathrm{Re}\{\mathcal{L}[G_\parallel(t)]_{\tilde{s}}\}. \tag{11}$$

With

$$\tau_\parallel = (q_\ell^2 D_\parallel)^{-1}, \tag{12}$$

the Laplace transform is given by

$$L \equiv \mathcal{L}\,[G_\parallel(t)]_{\tilde{s}} = \frac{1}{\Delta q}\left(\frac{\tau_\perp}{(1 + i\omega\tau_x)D_\parallel}\right)^{1/2} \left[\frac{\pi}{2} - \arctan\left(\frac{\tau_x}{(1 + i\omega\tau_x)\,\tau_\parallel}\right)^{1/2}\right], \tag{13}$$

where we have first carried out the transformation and then the integration. Moreover we have replaced the upper integration limit by ∞ for simplicity. In our frequency range this does certainly not affect the results.

In the high-frequency limit, $\omega\tau_x \gg 1$, we have

$$L_h \approx \frac{q_\ell}{\Delta q}\left(\frac{\tau_\parallel}{i\omega}\right)^{1/2}\left[\frac{\pi}{2} - \arctan\,(i\omega\tau_\parallel)^{-1/2}\right] \tag{14}$$

i.e., we can neglect the influence of the tumbling and exchange rates. In the low-frequency limit, $\omega\tau_x \ll 1$, a frequency independent expression comes out:

$$L_\ell \approx \frac{q_\ell}{\Delta q}(\tau_\parallel\,\tau_x)^{1/2}\left[\frac{\pi}{2} - \arctan\left(\frac{\tau_x}{\tau_\parallel}\right)^{1/2}\right] \tag{15}$$

The corresponding intensity functions for the high and low frequency limits are

$$I_h(\omega) \approx \frac{a_2}{\Delta q}(2D_\parallel\omega)^{-1/2}\left[\pi - \ln\frac{1-\sqrt{2}\,X_\xi + X_\xi^2}{1+\sqrt{2}\,X_\xi + X_\xi^2} - \arctan(\sqrt{2}\,X_\xi - 1) - \arctan(\sqrt{2}\,X_\xi + 1)\right], (\omega\tau_x \gg 1) \quad (16)$$

with

$$X_\xi = (\omega\tau_\parallel)^{-1/2} \text{ and } I_\xi(\omega) \approx \frac{2a_2}{\Delta q}\left(\frac{\tau_x}{D_\parallel}\right)^{1/2} \cdot \left[\frac{\pi}{2} - \arctan\left(\frac{\tau_x}{\tau_\parallel}\right)^{1/2}\right], (\omega\tau_x \ll 1) \quad (17)$$

respectively. Eq. 17 can be further approximated by

$$I_\xi(\omega) \approx \frac{2a_2}{\Delta q}\left(\frac{\tau_x}{D_\parallel}\right)^{1/2}\left[1 - \frac{2}{\pi}\left(\frac{\tau_x}{D_\parallel}\right)^{1/2}\right] \quad \text{for } \tau_x \ll \tau_\parallel \quad (18)$$

and

$$I_\xi(\omega) \approx \frac{2a_2}{\Delta q}\left(\frac{\tau_\parallel}{D_\parallel}\right)^{1/2} \quad \text{for } \tau_x \gg \tau_\parallel. \quad (19)$$

In the extreme limit of Eq. 18 the bracket term can be replaced by 1. Interestingly the limiting expressions then have a similar form. The constants τ_x and $\tau_\parallel$ are simply interchanged, while the prefactors have values not far from each other.

Designating the fraction of free water by p_f and spin-lattice relaxation rate of the free water by $(T_1^f)^{-1}$ and that of the hydration water by $(T_1^h)^{-1}$, we obtain the two-site exchange formula

$$\frac{1}{T_1} = \frac{1-p_f}{T_1^h} + \frac{p_f}{T_1^f}, \quad (20)$$

where T_1^f is constant within our frequency range. The relaxation rate of the hydration water is given by (13, p. 314)

$$\frac{1}{T_1^h} = C\,[I(\omega) + 4I(2\omega)], \quad (21)$$

where C is determined by the quadrupole coupling constant and $I(\omega)$ is the effective intensity function.

On the basis of Eqs. 18 and 19 one expects a low-frequency plateau in the T_1-dispersion beginning at an "inflection frequency"

$$\nu_i = \begin{cases}(2\pi\tau_x)^{-1} & \text{for } \tau_x \ll \tau_\parallel \\ (2\pi\tau_\parallel)^{-1} & \text{for } \tau_x \gg \tau_\parallel.\end{cases} \quad (22)$$

Thus, for fitting purposes, we can formally replace $I(\omega)$ in Eq. 21 by the limiting expression 16 both at low and high frequencies by introducing a new parameter

$$\tilde{x} = \left(\frac{\nu_i}{\nu}\right)^{1/2} \quad (23)$$

instead of X_ξ. In order to avoid confusion, we designate the new quantities in Eq. 21 by $\tilde{C}$ and $\tilde{I}(\omega)$, respectively.

In total a four-parameter formalism has been developed. The complete set of formulae is

$$\frac{1}{T_1} = \frac{1-p_f}{T_1^h} + \frac{p_f}{T_1^f}, \quad (24a)$$

$$\frac{1}{T_1^h} = \tilde{C}\,[\tilde{I}(\omega) + 4\tilde{I}(2\omega)], \quad (24b)$$

$$\tilde{I}(\omega) = \frac{1}{\sqrt{\omega}}\left[\pi - \ln\frac{1-\sqrt{2}\,\tilde{x} + \tilde{x}^2}{1+\sqrt{2}\,\tilde{x} + \tilde{x}^2} - \arctan(\sqrt{2}\,\tilde{x} - 1) - \arctan(\sqrt{2}\,\tilde{x} + 1)\right], \quad (24c)$$

$$\tilde{C} = C\,a_2\,\Delta q^{-1}\,(2D_\parallel)^{-1/2}. \quad (24d)$$

The parameters to be fitted in principle are p_f, $\tilde{C}$, ν_i, and T_1^f. Depending on the experimental data, situations can arise where the fits of these parameters are not unambiguous. In order to be sure that ambiguities of that kind can be excluded, we replace the above quantities by a set of three new parameters which are certainly independent from each other:

$$k_1 = \nu_i, \quad (25a)$$

$$k_2 = (1 - p_f)\,\tilde{C}, \quad (25b)$$

$$k_3 = p_f/T_1^f. \quad (25c)$$

RESULTS AND CONSISTENCY CHECKS

The field-cycling relaxation data are plotted in Figs. 1–3. Two proteins of different molecular sizes have been studied at two temperatures. With one of the proteins the water concentration has been varied as a parameter. The solid lines in the plots represent the theoretical formalism according to Eqs. (24a–d).

The parameters partly depend on the water concentration. Partly they are related to each other for theoretical reasons. In this section we present interpretations of these findings. Simultaneously the descriptions of the parameter dependences have the quality of consistency checks of the whole concept.

The fraction of free water, p_f, is in principle composed of two contributions. At high water concentrations, C_w, there will be an excess amount of free water in addition to thermally activated water. Designating the concentration where the hydration shells begin to overlap by C_o, we distinguish the concentration regimes $C_w < C_o$, where thermal activation is relevant alone and $C_w > C_o$, where excess water exists in addition. Assuming a Boltzmann

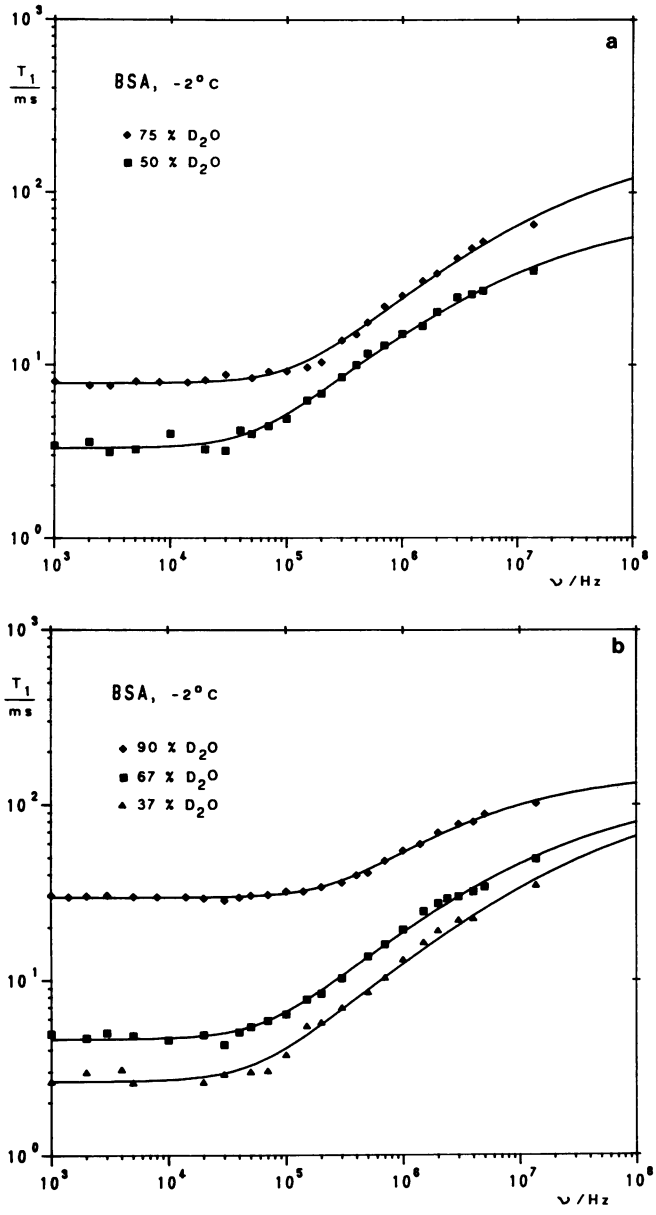

FIGURE 1 Deuteron-T_1-dispersion of BSA hydrated with D_2O at −2°C.
(*a*) C_w = 50% and 75%.
(*b*) C_w = 37%, 67%, and 90%.
The theoretical curves represent fits of Eq. 24, a–c on the basis of the parameters Eq. (25, a–c).

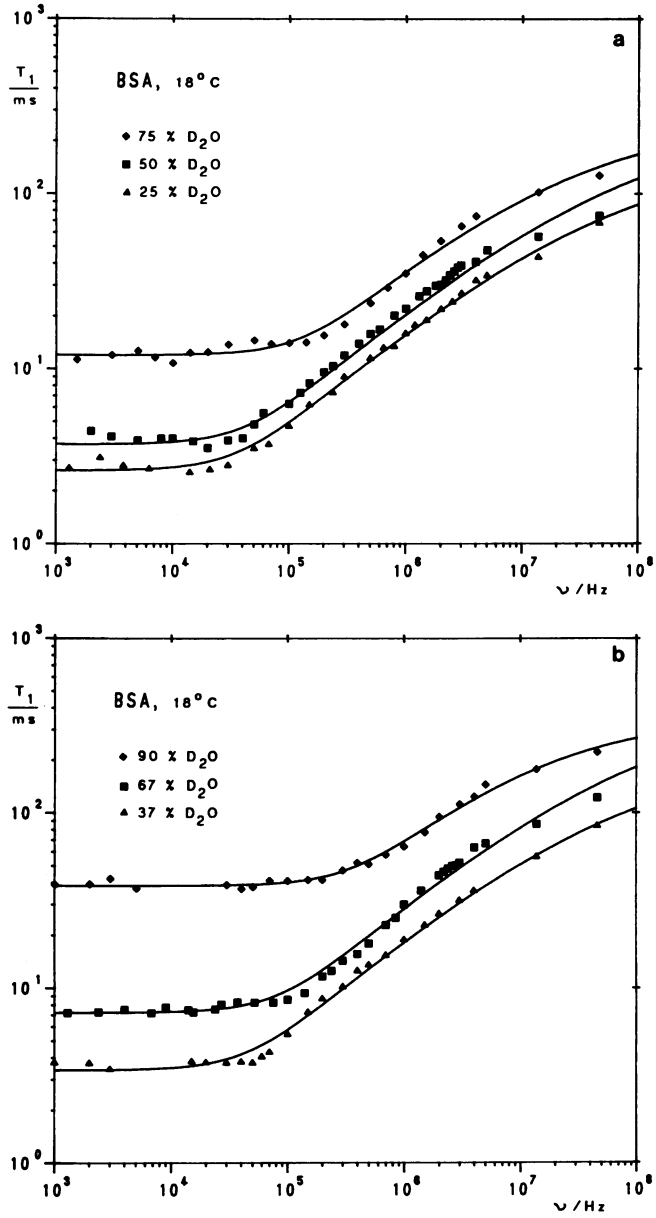

FIGURE 2 Deuteron-T_1-dipersion of BSA hydrated with D_2O at 18°C.
(*a*) C_w = 25%, 50%, and 75%.
(*b*) C_w = 37% , 67%, and 90%.
The theoretical curves represent fits of Eq. 24, a–c on the basis of the parameters Eq. 25, a–c.

distribution for the activated water, we have

$$p_f = [1 + \exp(\Delta G/RT)]^{-1} \text{ for } C_w \leq C_o \quad (26)$$

and

$$p_f = \frac{1}{1 - C_o}\left[\frac{C_w - C_o}{C_w} + \frac{C_o}{C_w}(1 - C_w)\frac{1}{1 + \exp(\Delta G/RT)}\right] \text{ for } C_w \geq C_o. \quad (27)$$

ΔG is the difference in the Gibbs free energy for 1 mol water between the hydration and the free state.

The overlap concentration, C_o, enters in a second way via the dependence of the inflection frequencey ν_i on p_f. As the tumbling rate and the rate for exchange between hydration and free water depends on the fraction of free water, we have to distinguish again two concentration regimes

$$\nu_i = (2\pi\tau_\parallel)^{-1} \text{ for } C_w < C_o \quad (28)$$

and

$$\nu_i = (2\pi\tau_x)^{-1} \text{ for } C_w > C_o. \quad (29)$$

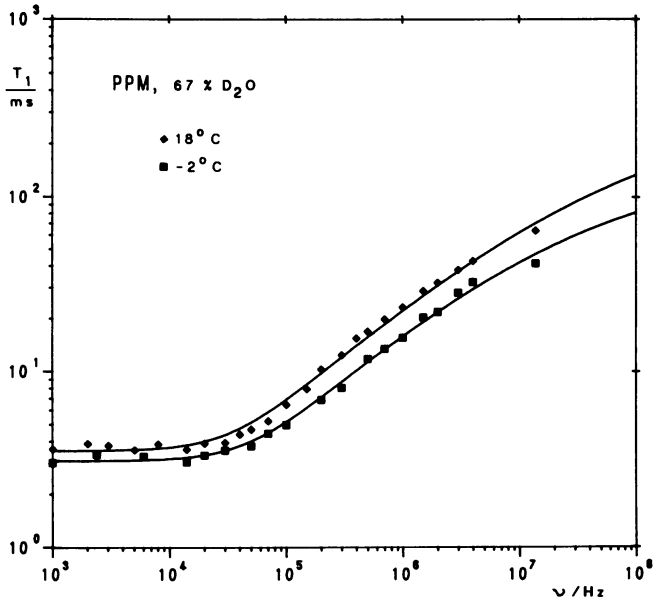

FIGURE 3 Deuteron-T_1-dispersion of purple membrane sheets hydrated with 67% D_2O at −2°C and 18°C. The theoretical curves represent fits of Eq. 24, a–c on the basis of the parameters Eq. 25, a–c. The parameters are $k_1 = \nu_i = 8.0 \cdot 10^4$ Hz, $k_2 = 1.06 \cdot 10^4\ s^{-1/2}$, $k_3 = 7.17\ s^{-1}$ and $k_1 = \nu_i = 5.8 \cdot 10^4$ Hz, $k_2 = 7.9 \cdot 10^3\ s^{-1/2}$, $k_3 = 3.6\ s^{-1}$ for −2°C and 18°C, respectively.

The tumbling of the whole macromolecules is only possible if sufficient free water "lubricates" this kind of motion. The Stokes-Einstein-law is expected to be valid,

$$\tau_t = \frac{4\pi\eta r^3}{k_B T} \tag{30}$$

with the microviscosity η and the radius r of the hydrated macromolecule (assumed spherical). k_B is Boltzmann's constant. There is no theory available relating the microviscosity with the water concentration or p_f. In any case, η must decrease with p_f, and we will use

$$\eta = \frac{\eta_0}{p_f} \tag{31}$$

or

$$\tau_t = \tau_t^0 p_f^{-1}, \tag{32}$$

where η_0 is the viscosity of free water and τ_t^0 the tumbling time in the limit $p_f \to 1$.

The exchange between hydration and free water also depends on the existence of both phases. Let us again assume a proportionality. We write

$$\frac{1}{\tau_\perp} = \frac{p_f}{\tau_\perp^0}, \tag{33}$$

where τ_0 is the time which would be needed for the exchange between both phases by perpendicular diffusion in the limit $p_f \to 1$.

Combining Eqs. 32 and 33 with Eq. 10 leads to

$$\frac{1}{\tau_x} = \frac{p_f}{\tau_x^0} \tag{34}$$

τ_x^0 will be governed by τ_t^0 if $\tau_t^0 \ll \tau_\perp^0$ and by $\tau_\perp^0$ if $\tau_t^0 \gg \tau_\perp^0$. The validity of one of these limits depends on the actual conditions of the system. A discussion follows below.

Inserting Eqs. 26 and 27 into Eqs. 33 and 34 leads to the concentration dependence of ν_i (Eqs. 28 and 29). Figs. 4 *a* and *b* show the fits of these expressions to the ν_i values deduced from the T_1-dispersion curves. The coincidence is almost perfect. Moreover an overlap concentration $C_0 \approx 65\%$ has been found. Note that this concentration is defined by weight and refers to D_2O. The corresponding value, for H_2O would be somewhat lower.

A further consistency check refers to the relation between the low-frequency plateau relaxation time T_1^p and the inflection frequency ν_i. From Eqs. 18, 19, and 24 it follows that

$$k_2\left(\frac{1}{T_1^p} - k_3\right)^{-1} = \tilde{C}(1 - p_f)\left(\frac{1}{T_1^p} - \frac{p_f}{T_1^f}\right)^{-1} \sim (\nu_i)^{1/2}, \tag{35}$$

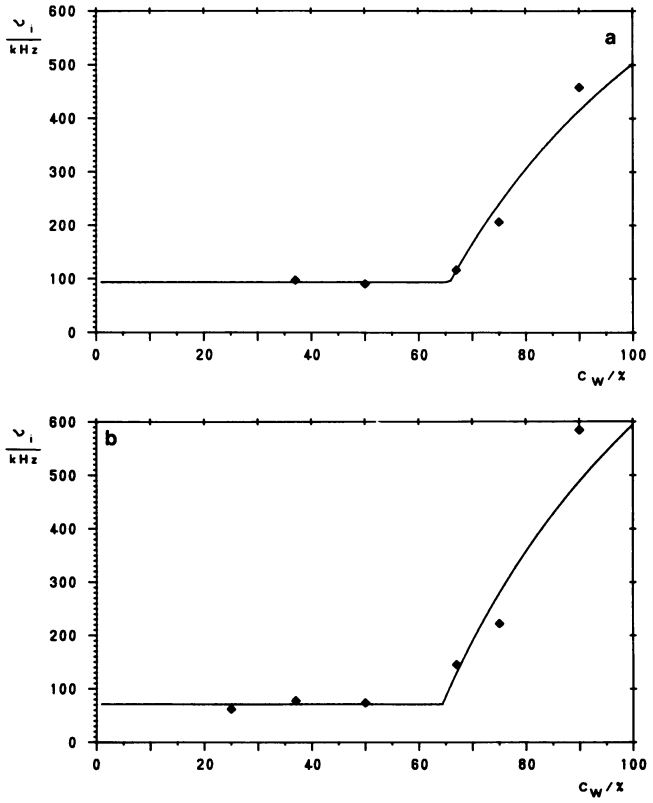

FIGURE 4 Inflection frequency versus water content for BSA at −2°C (*a*) and 18°C (*b*). The solid lines represent fits of Eqs. 27–29 and 34 to the ν_i-values derived from the T_1-dispersion curves. The parameters are $C_0 = 65\%$, $\tau_x^0 = 3.2 \cdot 10^{-7}$ s, $\tau_\parallel = 1.7 \cdot 10^{-6}$ s, $\Delta G = 3.8 \cdot$ kJ/mol and $C_0 = 64\%$, $\tau_x^0 = 2.7 \cdot 10^{-7}$ s, $\tau_\parallel = 2.2 \cdot 10^{-6}$ s, $\Delta G = 8.7$ kJ/mol for −2°C and 18°C, respectively.

where the k_3 term is negligible for low water concentrations.

We abbreviate the left-hand side of Eq. 35 by k_4. This quantity is plotted versus ν_i in Figs. 5 *a* and *b*. Obviously the data can well be described by the above proportionality.

In Figs. 6 *a* and *b* we have plotted the parameter k_2 versus C_w. A break is visible which again can be attributed to the crossover at $C_w = C_0 \approx 70\%$. Inserting Eqs. 26 and 27 for p_f in the expression (25b) and assuming a relation

$$\tilde{C} = KC_w^{-x} \tag{36}$$

leads to the reasonable description of the data in Figs. 6 *a* and *b* (solid lines). According to Eq. (24d) we have $\tilde{C} \sim D_\parallel^{-1/2}$, i.e., together with Eq. 36 $D_\parallel \sim C_w^{2x}$. From the fits, x turns out to be roughly $x \approx 0.5$, so that $D_\parallel \sim C_w$. This conclusion does not appear to be unreasonable, but we have no detailed interpretation of it. Further results of these fits are $\Delta G = 5.5$ kJ/mol, $K = 7.9 \cdot 10^3$ s$^{-1/2}$ and $\Delta G = 5.1$ kJ/mol, $K = 6.8 \cdot 10^3$ s$^{-1/2}$ for -2°C and 18°C, respectively. The ΔG values are not far from those obtained from the independent fit represented by Fig. 4.

The state of the free water is such that we can assume the extreme narrowing condition $\omega\tau_c \ll 1$, where τ_c is the correlation time effective in the free water. Hence $T_1^f \sim \tau_c^{-1}$ and, because of $\tau_c \sim \eta$, $T_1^f \sim \eta^{-1}$. According to Eq. 31 we use $\eta^{-1} \sim p_f$ and find

$$k_3 = \frac{p_f}{T_1^f} = \text{const} \tag{37}$$

The parameter k_3 should be independent of C_w. Unfortunately it is completely determined by the tendency of the high-frequency T_1 data (Figs. 1–3) to form a plateau. This is however not very distinct in our frequency range, and the fitted values of k_3 hence scatter about the mean within ±50%. Nevertheless, we can state that there can be no strong deviation from the constancy expressed by Eq. 37.

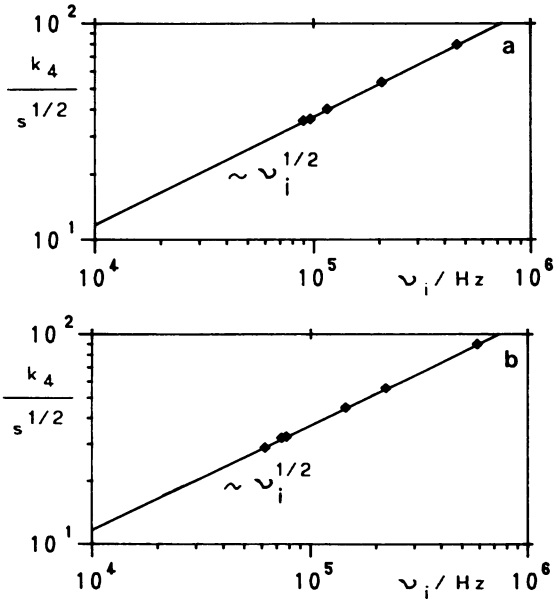

FIGURE 5 Verification on Eq. 35 for BSA. (*a*) −2°C, (*b*) 18°C. (k_4 is identical to the lefthand side of Eq. 35).

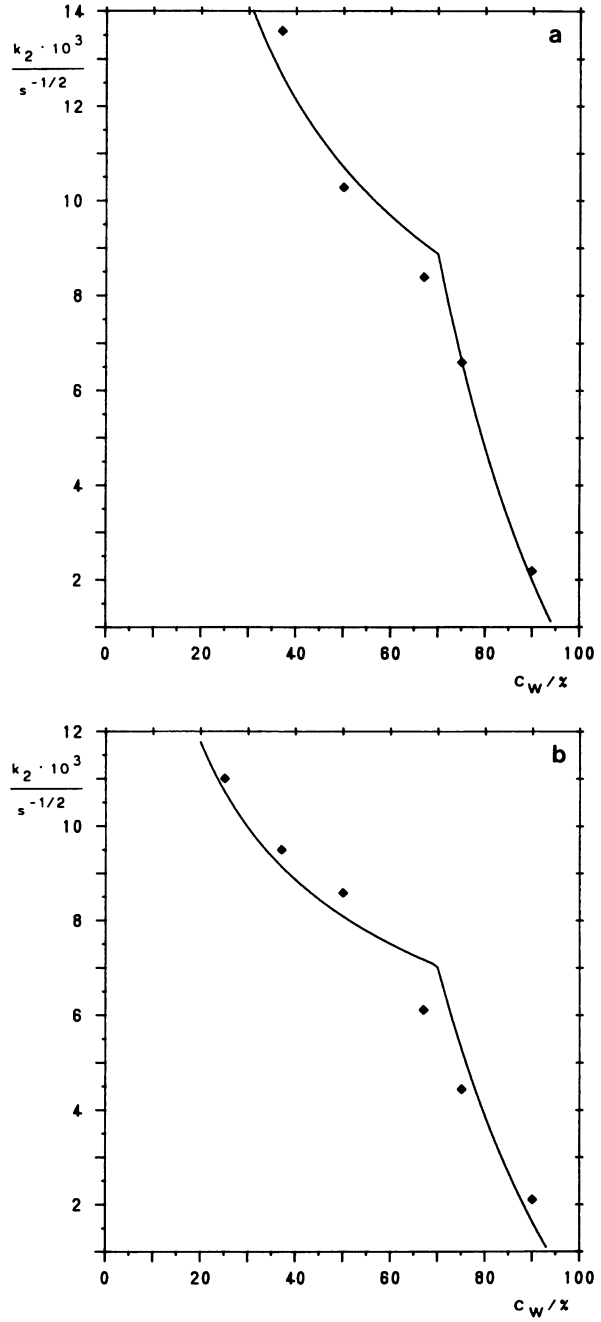

FIGURE 6 Parameter k_2 (Eq. 25b) versus water content for BSA at −2°C (*a*) and 18°C (*b*).

DISCUSSION

It has been shown that the experimental data can consistently be described by the translational diffusion concept. Let us now discuss the absolute values of the fitted parameters.

The thickness of the hydration shells effective for the

deuteron relaxation can be estimated on the basis of the geometrical data of BSA molecules (14). Taking a density of 1 g cm^{-3} both for water and protein for simplicity, one estimates a thickness $d \approx 7$ Å at the overlap concentration $C_o \approx 65\%$. This corresponds to roughly two monomolecular layers of water molecules if one assumes a closed coverage of the protein surface. Thus, all water molecules outside of the two monomolecular layers are classified as free.

The problem of surface-induced perturbation has been discussed in reference 15 from a more general point of view. The same effective thickness of the hydration shell has been concluded. In reference 9 the dependence of ν_i on the molecular weight M has been studied in dilute protein solutions. The data could well be described by $\nu_i \sim M^{-1}$ as suggested by Eq. 29. We conclude that the exchange rate $\tau_\perp^{-1}$ is negligible compared with the tumbling rate τ_t^{-1} so that $\tau_x \approx \tau_t$ for $C_w > C_o$. Such long exchange times are consistent with the slow proton exchange in neutral water discussed e.g., in reference 16. Note, however, that the above conclusion implies that the exchange of whole water molecules is also sufficiently slow. In total we expect $\tau_t < \tau_\perp < T_1$ for $C_w > C_o$.

At low water concentrations, $C_w < C_o$, where free tumbling becomes impossible, the exchange rate $\tau_\perp^{-1}$ should again be negligible under the conditions of the present experiments, and we assume $\tau_x \approx \tau_\parallel$ or $\tau_\parallel < \tau_\perp < T_1$ for $C_w < C_o$. There are two reasons for this limit. First, if $\tau_\perp^{-1}$ would dominate, a concentration dependence of γ_i e.g., according to Eq. 33 would arise, what has not been observed (Fig. 4). Second, $\tau_\perp$ must be independent of the shape or the size of the protein aggregates. The finding is, however, that purple membrane sheets have τ_x-times longer by a factor of 2 than BSA forming not such aggregates. Thus $\tau_\parallel$ can be determined from ν_i for low water concentrations.

Tentatively characterizing the dimensions of a BSA molecule (14) by the circumference (~300 Å) and assuming that the lower limit of the wave number is determined by this length, one estimates from Eq. 12 $D_\parallel \approx 10^{-7}$ cm^2 s^{-1} as an average over the first two monomolecular layers. In reference 17 a higher value has been reported. This was, however, determined as an average over a 10 Å layer about the protein, so that "free water" should have had a strong influence. Note also, that in the present study much higher values of the mean square displacement are relevant. Thus waiting time delays at certain binding sites could slow down the displacement rates. Possibly neutron scattering studies (18) would provide additional insight into this problem.

Furthermore, the above value for $D_\parallel$ tends to be underestimated. At low water concentrations an overlap of the hydration shells of neighboring protein molecules occurs. In a sense, we are dealing with a percolation problem. The long-wave-length cut off may then be expected to be not determined by the protein dimensions any more, and the situation approaches that of purple membrane sheets.

The hydration shell mechanism introduced above intrinsically implies a $\nu^{1/2}$-dispersion of T_1. Such a behavior has already been discussed (19,20) from different viewpoints. Note also, that the central point of our concept, namely translational diffusion, in literature is often used in the sense of modulations of internuclear distances (21). These are, however, irrelevant for deuteron relaxation.

The frequency dependence of proton relaxation times is certainly influenced by additional effects. Fluctuations within the protein (27) are expected to play a role via cross-relaxation (4). Thus, the effective proton relaxation behavior must be traced back to several sources.

In reference 28 it has been suggested that chemical exchange with protein hydrogens significantly contributes to the total relaxation rate. Moreover, a mutual interference of hydration water and protein motions has been proposed (29). On the other hand, protons of D_2O-hydrated and dry proteins show the same T_1-dispersion, while the proton T_1-dispersion of H_2O-hydrated proteins differs from that of the dry proteins (4). The latter finding indicates, that one can in fact distinguish a proton phase in connection with hydration and another one related to the protein skeleton. Thus we believe, that a unified relaxation theory can be composed of the "elements" mentioned above.

A basic prerequisite of our interpretation scheme is the existence of orientational order of hydration water with respect to the protein surface. Many investigations have been reported about this subject (e.g., 22–26). In summary, one may conclude that there can be no doubt about the anisotropic behavior of surface water. Let us finally draw the attention to an interesting analogy to other types of ordered structures and their fluctuations (30,31). Provided that something like a "rugged" surface structure exists, our formalism should also be applicable to quite different systems such as lamellae or micellar phases.

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