

# A New View of Water Dynamics in Immobilized Proteins

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**ABSTRACT** The inflection frequency of the deuteron magnetic relaxation dispersion from water in rotationally immobilized protein samples has recently been found to be essentially independent of temperature and protein structure. This remarkable invariance has been interpreted in terms of a universal residence time of 1  $\mu$ s for protein-associated water molecules. We demonstrate here that this interpretation is an artifact of the conventional perturbation theory of spin relaxation, which is not valid for rotationally immobile proteins. Using a newly developed non-perturbative, stochastic theory of spin relaxation, we identify the apparent correlation time of 1  $\mu$ s with the inverse of the nuclear quadrupole frequency, thus explaining its invariance. The observed dispersion profiles are consistent with a broad distribution of residence times, spanning the  $\mu$ s range. Furthermore, we argue that the deuteron dispersion is due to buried water molecules rather than to the traditional surface hydration previously invoked, and that the contribution from rapidly exchanging protein hydrogens cannot be neglected. The conclusions of the present work are also relevant to proton relaxation in immobilized protein samples and to magnetic resonance imaging of soft tissue.

## INTRODUCTION

The Larmor frequency dispersion of the longitudinal spin relaxation rate  $R_1$  of the water nuclei  $^1\text{H}$ ,  $^2\text{H}$ , and  $^{17}\text{O}$  has been widely used to characterize the dynamics of water molecules in aqueous protein solutions (Koenig and Brown, 1991; Belton, 1994). A variety of models for the hydration dynamics have been introduced to account for the  $R_1$  dispersion profiles. The dynamic models postulated by different authors include specific long-lived ( $\sim 10^{-6}$  s) hydration sites at the protein surface (Koenig and Schillinger, 1969; Koenig et al., 1993b); a relatively long-lived ( $> 10^{-8}$  s), locally anisotropic hydration layer (Halle et al., 1981; Halle and Piculell, 1986); a very long-lived ( $> 10^{-6}$  s) hydration layer with faster lateral diffusion over the protein surface (Schauer et al., 1988; Kimmich et al., 1990); and a long-ranged hydrodynamic coupling between protein and water rotation (Koenig et al., 1975; Koenig and Brown, 1991). While all of these mutually conflicting models (and some more) have been claimed to account for the measured  $R_1$  dispersion profiles, none of them has been subjected to a decisive experimental test.

We have recently carried out systematic studies of the water  $^{17}\text{O}$  and  $^2\text{H}$  relaxation dispersion profiles from aqueous protein solutions, with the aim of resolving the long-standing interpretational controversy (Denisov and Halle, 1994; Denisov and Halle, 1995a, b; V. Denisov, submitted for publication). Our hypothesis was that the relaxation dispersion is due not to water molecules residing at the

protein surface as previously assumed, but rather to a small number of water molecules buried inside the protein. Drawing on the extensive amount of high-resolution protein crystal structure data deposited in the Protein Data Bank, including the number and location of buried water molecules, we could decisively test the hypothesis. The results of these studies unambiguously identify the origin of the  $^{17}\text{O}$  relaxation dispersion as buried water molecules exchanging with bulk water on the time scale  $10^{-8}$ – $10^{-6}$  s, presumably via conformational fluctuations of the kind invoked for amide hydrogen exchange (Englander and Kallenbach, 1984). Because of the smaller nuclear quadrupole frequency of the deuteron, the  $^2\text{H}$  dispersion monitors buried water molecules exchanging on the broader time scale  $10^{-8}$ – $10^{-4}$  s, and, depending on pH, may also include a significant contribution from labile protein hydrogens exchanging on this time scale (Denisov and Halle, 1995b). Under typical solution conditions, the rotational Brownian motion of the protein molecule averages to zero the anisotropic quadrupole coupling experienced by the  $^2\text{H}$  or  $^{17}\text{O}$  nucleus in a buried water molecule. Consequently, the inflection frequency of the relaxation dispersion should correspond to the rotational correlation time of the protein, as has been amply verified (Koenig and Brown, 1991; Belton, 1994).

In recent years, several groups have measured the  $^2\text{H}$  relaxation dispersion in aqueous protein samples, where the native protein molecules are prevented from rotating by chemical cross-linking (Koenig et al., 1993b; Koenig and Brown, 1993) or by non-covalent interactions at high (typically  $> 50$  wt %) protein concentration (Kimmich et al., 1990). Such rotationally immobilized protein samples are not only useful for elucidating the molecular mechanism behind the relaxation dispersion, but may also serve as models for water relaxation in soft tissue (Brown and Koenig, 1992; Koenig and Brown, 1994). In such samples, the inflection frequency should reflect not protein rotation (which is inhibited), but the actual residence times of buried

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water molecules (and, possibly, labile protein hydrogens). From our recent studies of bovine pancreatic trypsin inhibitor (BPTI), we know that these residence times can be of the same order as, and longer than, the inverse of the deuteron quadrupole frequency,  $\sim 10^{-6}$  s (Denisov and Halle, 1995a, b; Denisov et al., 1995). Under such conditions, the conventional second-order perturbation theory of spin relaxation breaks down (Abragam, 1961) and a more general approach is required. Motivated by this problem, we have recently developed a stochastic theory of spin relaxation (B. Halle, submitted for publication) based on an analytical solution of the stochastic Liouville equation. This theory is directly applicable to the relaxation dispersion of quadrupolar nuclei in rotationally immobilized protein samples. In the present work, we apply this generalized relaxation theory to  $^2\text{H}$  relaxation dispersion data from chemically cross-linked bovine serum albumin (BSA) (Koenig et al., 1993b; Koenig and Brown, 1993). Our conclusions should also apply to  $^2\text{H}$  relaxation dispersion data from highly concentrated BSA solutions (Kimmich et al., 1990).

In both chemically cross-linked and highly concentrated BSA solutions, the  $R_1$  dispersion is dominated by a large dispersion step with an inflection frequency corresponding to a correlation time of  $\sim 10^{-6}$  s. Both groups based their interpretation on the conventional perturbation theory of spin relaxation, but arrived at radically different conclusions, because they postulated different models for the hydration dynamics. Koenig and colleagues ascribe the major dispersion to eight strongly hydrogen-bonded water molecules at specific sites on the protein surface (this was later revised to two water molecules (Koenig and Brown, 1993, 1994)), all having a residence time of 1  $\mu\text{s}$  (Koenig et al., 1993b). A remarkable feature of this interpretation is that the residence time (as derived from the inflection frequency of the dispersion profile) of these special surface waters appears to have a universal value of 1  $\mu\text{s}$  for different proteins and at different temperatures (Koenig et al., 1993b). Kimmich et al. (1990) rationalize their very similar dispersion profiles in terms of lateral diffusion, on the  $\mu\text{s}$  time scale, of all water molecules in the first hydration layer, implying that the residence times for the  $>1000$  surface waters are longer than  $10^{-6}$  s.

Analyzing the  $^2\text{H}$  relaxation dispersion data from cross-linked BSA in terms of the generalized relaxation theory, we find that the apparent correlation time of 1  $\mu\text{s}$  is essentially the inverse of the nuclear quadrupole frequency and, hence, is not directly related to water dynamics. According to the theory, any distribution of residence times that spans the  $\mu\text{s}$  range results in a relaxation dispersion with an inflection frequency corresponding to the inverse quadrupole frequency. This finding provides a simple explanation for the universality of the apparent correlation time. We also conclude that the available  $^2\text{H}$  relaxation dispersion data from immobilized protein samples can be accounted for in terms of exchanging buried water molecules and labile protein hydrogens, as previously established for protein solutions (Denisov and Halle, 1995a, b).

## THEORY

### Relaxation dispersion in protein solutions

The longitudinal relaxation rate  $R_1$  of the observed bulk water  $^2\text{H}$  resonance in a solution of rotationally mobile protein molecules can be expressed, within the framework of conventional spin relaxation theory (Abragam, 1961), as follows (Denisov and Halle, 1995a, b):

$$R_1(\omega_L) = (1 - f_S)R_{\text{bulk}} + f_S\langle R_S \rangle + f_I\langle R_I(\omega_L) \rangle. \quad (1)$$

Here  $R_{\text{bulk}}$  is the known bulk water relaxation rate,  $\langle R_S \rangle$  is the average relaxation rate for the fraction  $f_S$  of deuterons residing in water molecules whose rotational motion is significantly perturbed by the protein surface, and  $\langle R_I(\omega_L) \rangle$  is the average relaxation rate for the very small fraction  $f_I$  of deuterons residing in buried water molecules or in labile protein hydrogens with residence times shorter than  $1/R_1$ . Note that the dependence on the Larmor frequency  $\omega_L$ , i.e., the relaxation dispersion, is due entirely to the "internal" deuterons, represented by the last term in Eq. 1.

If all internal deuterons have residence times,  $\tau_{\text{res}}$ , longer than the rotational correlation time,  $\tau_R$ , of the protein, the dispersive contribution can be expressed as

$$\langle R_I(\omega_L) \rangle = 2J(\omega_L) + 8J(2\omega_L), \quad (2)$$

with the Lorentzian spectral density function

$$J(m\omega_L) = \frac{1}{15} \langle \omega_Q^2 \rangle \frac{\tau_R}{1 + (m\omega_L\tau_R)^2}. \quad (3)$$

Here we have introduced the residual nuclear quadrupole frequency

$$\omega_Q = \frac{3\pi}{2} A \chi, \quad (4)$$

with  $\chi$  the usual quadrupole coupling constant (Abragam, 1961) and  $A$  the generalized order parameter (Halle and Wennerström, 1981). For the deuterons under consideration,  $A$  reduces to the usual second-rank orientational order parameter, which varies from 0 for an isotropically disordered O— $^2\text{H}$  (or N— $^2\text{H}$ ) bond to 1 for a perfectly ordered bond. The average in Eq. 3 is over all internal deuterons that contribute to the dispersion and thus have residence times in the range (Denisov and Halle, 1995a, b)

$$\tau_R < \tau_{\text{res}} < [R_I(0)]^{-1}. \quad (5)$$

The theoretical framework defined by Eqs. 1–5 quantitatively accounts for the  $^2\text{H}$  (and  $^{17}\text{O}$ ) relaxation dispersion from buried water molecules in protein solutions (Denisov and Halle, 1995a, b; V. Denisov, submitted for publication).

### Relaxation dispersion in rotationally immobilized protein samples

If protein rotation is inhibited, the correlation time in the spectral density function, Eq. 3, can no longer be the rota-

tional correlation time  $\tau_R$ . Because the fraction  $f_i$  of internal deuterons is very small, the mean time,  $\tau_{\text{res}}/f_i$ , during which a water molecule diffuses between two successive visits to internal sites is sufficiently long that, after leaving a given internal site, a water molecule can reach any other internal site (on the same protein molecule or on a different one) with essentially equal probability. Given that the immobilized protein samples under consideration (unlike protein crystals) are macroscopically isotropic, it then follows that each exchange event brings about complete orientational randomization of the anisotropic quadrupole coupling experienced by an internal deuteron. The residence time  $\tau_{\text{res}}$  then becomes the correlation time and Eq. 3 should be replaced by

$$J(m\omega_L) = \frac{1}{15} \left\langle \frac{\omega_Q^2 \tau_{\text{res}}}{1 + (m\omega_L \tau_{\text{res}})^2} \right\rangle, \quad (6)$$

where, as before, the average is over all internal deuterons that contribute to the dispersion. Note that this spectral density function is, in general, a superposition of Lorentzians.

The spectral density function in Eq. 6 is valid only within the motional-narrowing regime,

$$(\omega_Q \tau_{\text{res}})^2 \ll 1 + (\omega_L \tau_{\text{res}})^2, \quad (7)$$

where the conventional perturbation theory of spin relaxation applies (Abragam, 1961). Since the residual quadrupole frequency  $\omega_Q$  is  $\sim 10^6 \text{ rad s}^{-1}$  (cf. below), the motional-narrowing condition, Eq. 7, is violated for residence times of order  $10^{-6} \text{ s}$  or longer. Considering that the inflection frequency of the experimental relaxation profiles is near  $10^6 \text{ rad s}^{-1}$ , we clearly need a more general description of spin relaxation than that provided by the conventional perturbation theory. Such a generalized relaxation theory has recently been developed (B. Halle, submitted for publication). In the experimentally relevant dilute regime ( $f_i \ll 1$ ), this theory predicts that the longitudinal  $^2\text{H}$  relaxation is exponential (as observed) with the relaxation rate obtained from Eqs. 1 and 2, but with the spectral density function in Eq. 6 replaced by the generalized spectral density function (B. Halle, submitted for publication)

$$\tilde{J}(m\omega_L) = \frac{1}{15} \left\langle \frac{\omega_Q^2 \tau_{\text{res}}}{1 + (\omega_Q \tau_{\text{res}})^2 + (m\omega_L \tau_{\text{res}})^2} \right\rangle. \quad (8)$$

As expected, this reduces to Eq. 6 when the motional-narrowing condition, Eq. 7, is satisfied. It should be noted that Eq. 8 is not subject to any restrictions on the values of  $\omega_L$ ,  $\omega_Q$ , and  $\tau_{\text{res}}$ .

It is instructive to cast Eq. 8 on the form of the motional-narrowing spectral density, Eq. 6, as

$$f_i \tilde{J}(m\omega_L) = \frac{1}{15} \left\langle \frac{\hat{f}_i \omega_Q^2 \hat{\tau}_{\text{res}}}{1 + (m\omega_L \hat{\tau}_{\text{res}})^2} \right\rangle, \quad (9)$$

with the apparent internal-deuteron fraction  $\hat{f}_i$  and the apparent residence time  $\hat{\tau}_{\text{res}}$  given by

$$\hat{f}_i = f_i [1 + (\omega_Q \tau_{\text{res}})^2]^{-1/2}, \quad (10)$$

$$\hat{\tau}_{\text{res}} = \tau_{\text{res}} [1 + (\omega_Q \tau_{\text{res}})^2]^{-1/2}. \quad (11)$$

If Eq. 6 is used outside its domain of validity, the internal-deuteron fraction and residence time deduced from the dispersion profile are the apparent quantities in Eqs. 10 and 11. Eq. 11 shows that if  $\tau_{\text{res}} \gg 1/\omega_Q$ , then the apparent residence time  $\hat{\tau}_{\text{res}}$ , deduced from the dispersion profile using motional-narrowing theory, is nothing but the inverse of the nuclear quadrupole frequency, as given by Eq. 4.

For deuterons in buried water molecules, the residual quadrupole frequency,  $\omega_Q$ , should be close to  $10^6 \text{ rad s}^{-1}$  (cf. below), while the residence times,  $\tau_{\text{res}}$ , are expected to span a wide range. The average in Eq. 8 thus involves primarily the residence time distribution. Fig. 1 shows how deuterons with different residence times contribute to the magnitude of the dispersion step. The maximum contribution comes from  $\tau_{\text{res}} = 1/\omega_Q$ , and the relative contribution is reduced by a factor 5 (50) when  $\tau_{\text{res}}$  is shifted by 1 (2) decades away from  $1/\omega_Q$ . If there is a distribution of residence times, the relaxation dispersion will thus be dominated by deuterons with residence times near  $1/\omega_Q \approx 10^{-6} \text{ s}$ . In the following section, we demonstrate quantitatively that these theoretical considerations can account for the unexpected features of the  $^2\text{H}$  relaxation dispersion from immobilized protein samples.

## RESULTS AND DISCUSSION

### Motional-narrowing theory

We focus on the recently reported water  $^2\text{H}$  dispersion profile from chemically cross-linked BSA (Koenig et al., 1993b; Koenig and Brown, 1993). These data, reproduced in Fig. 2, refer to two different sets of experimental conditions, here denoted as samples A and B. Sample A contains 10 wt % BSA in a 54:46%  $\text{D}_2\text{O}:\text{H}_2\text{O}$  mixture at  $35^\circ\text{C}$

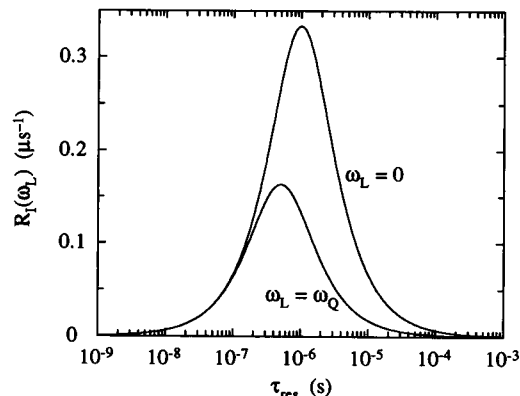


FIGURE 1 Contributions to the longitudinal relaxation rate,  $R_L$ , of internal deuterons with different residence times,  $\tau_{\text{res}}$ , and a quadrupole frequency of  $\omega_Q = 10^6 \text{ rad s}^{-1}$ .  $R_L$  was obtained from the generalized relaxation theory, according to Eqs. 2 and 8.

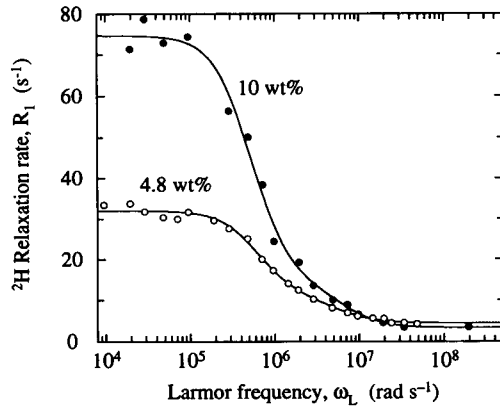


FIGURE 2 Longitudinal relaxation dispersion of water deuterons from chemically cross-linked BSA in sample A (10 wt %, 35°C) (Koenig et al., 1993b) and in sample B (4.8 wt %, 20°C) (Koenig and Brown, 1993). The curves resulted from fits of the five parameters (cf. Table 1) in Eq. 12.

(Koenig et al., 1993b), while sample B contains 4.8 wt % BSA in a 90:10% D<sub>2</sub>O:H<sub>2</sub>O mixture at 20°C (Koenig and Brown, 1993). In both samples, the nominal pH is 7 and the mol ratio cross-linking agent (glutaraldehyde)/BSA is ~80. Three main features are apparent from the data in Fig. 2; a major dispersion around  $\omega_L \approx 10^6$  rad s<sup>-1</sup>, a minor dispersion just below  $\omega_L \approx 10^7$  rad s<sup>-1</sup>, and a high-frequency plateau at  $\omega_L > 10^8$  rad s<sup>-1</sup>. The curves in Fig. 2 resulted from fits of the parameters in the expression

$$R_1(\omega_L) = \alpha + \beta_1 \tau_1 F(\omega_L \tau_1) + \beta_2 \tau_2 F(\omega_L \tau_2), \quad (12)$$

with

$$F(z) = \frac{2}{1+z^2} + \frac{8}{1+4z^2}. \quad (13)$$

The resulting values of the five parameters are given in Table 1 for both samples.

The physical significance of the parameters in Eq. 12 and Table 1 can be inferred by identification with the appropriate expressions in the Theory section. The parameter  $\alpha$ ,

**TABLE 1** Parameters and derived quantities from the fits of Eq. 12 to the relaxation dispersion data in Fig. 2

Quantity	Sample A	Sample B
Parameter from fit		
$\alpha$ (s <sup>-1</sup> )	$3 \pm 2$	$4.5 \pm 0.5$
$\beta_1$ (10 <sup>7</sup> s <sup>-2</sup> )	$1.1 \pm 0.7$	$0.6 \pm 0.2$
$\beta_2$ (10 <sup>7</sup> s <sup>-2</sup> )	$0.58 \pm 0.10$	$0.24 \pm 0.04$
$\tau_1$ (ns)	$90 \pm 60$	$100 \pm 40$
$\tau_2$ ( $\mu$ s)	$1.0 \pm 0.1$	$0.9 \pm 0.1$
Derived quantity		
$N_1$	$11 \pm 7$	$22 \pm 7$
$N_2$	$6 \pm 1$	$9 \pm 2$
$N$	$17 \pm 7$	$30 \pm 7$
$\eta$	$0.6 \pm 0.2$	$0.7 \pm 0.1$

describing the high-frequency plateau, corresponds to the first two terms in Eq. 1, i.e.,

$$\alpha = (1 - f_s)R_{\text{bulk}} + f_s \langle R_s \rangle. \quad (14)$$

The interpretation of the remaining four parameters depends on whether one adopts the conventional motional-narrowing theory or the generalized relaxation theory.

Within the conventional theory, the second and third terms in Eq. 12 can be interpreted in terms of a bimodal residence time distribution (Koenig et al., 1993b; Koenig and Brown, 1993). Alternatively, the minor dispersion could reflect the tumbling of BSA molecules in an incompletely cross-linked sample (Zhou and Bryant, 1994). Whichever interpretation is adopted, the numbers,  $N_1$  and  $N_2$ , of deuterons responsible for the minor and major dispersion steps can be obtained from the identification (cf. Eqs. 1, 2, and 6)

$$\beta_k = \frac{1}{15} f_{ik} \omega_Q^2 = \frac{N_k}{15x} \omega_Q^2, \quad (k = 1, 2). \quad (15)$$

From the sample composition and molecular weights, we obtain for the total number of water deuterons per BSA molecule  $x = 3.36 \times 10^4$  for sample A and  $x = 1.19 \times 10^5$  for sample B. For the residual quadrupole frequency, we use the value  $\omega_Q = 7 \times 10^5$  rad s<sup>-1</sup>. This corresponds to a plausible order parameter (Denisov and Halle, 1995a, b; Denisov, submitted for publication) of  $A = 0.70$  if the ice  $lh$  value of  $\chi = 213$  kHz (Edmonds and Mackay, 1975) is adopted for the quadrupole coupling constant. We thus obtain, from Eq. 15, the  $N_1$  and  $N_2$  values quoted in Table 1. According to this interpretation, the major dispersion is due to only three (4.5) water molecules per BSA molecule with a residence time of 1.0 (0.9)  $\mu$ s for sample A (B). On the basis of a somewhat different analysis, Koenig and colleagues arrived at eight (Koenig et al., 1993b) and two (Koenig and Brown, 1993, 1994) such water molecules for samples A and B, respectively. Furthermore, they assumed that these water molecules are strongly bound at the protein surface. If a bimodal residence time distribution is assumed, the minor dispersion would be due to 6 (sample A) or 11 (sample B) other water molecules with a residence time around 100 ns. For sample B, the minor dispersion was previously ascribed to 16 water molecules with a residence time of 23 ns (Koenig and Brown, 1993, 1994).

In the event of incomplete cross-linking, with a fraction  $\eta$  of tumbling BSA molecules,  $N_1$  should be multiplied by  $\eta$  and  $N_2$  by  $1 - \eta$  in Eq. 15. If the same (buried) water molecules are responsible for both dispersion steps ( $N_1 = N_2$ ), we obtain the  $N$  and  $\eta$  values quoted in Table 1. A recent study of the water <sup>1</sup>H relaxation dispersion in glutaraldehyde cross-linked BSA (8.25 wt %) shows a strong increase of the dispersion amplitude with the mol ratio glutaraldehyde/BSA, with no sign of saturation even at a mol ratio of 286 (Zhou and Bryant, 1994). Consequently, the samples discussed here, with a mol ratio of ~80, are surely not completely rotationally immobilized. The minor

dispersion can thus be ascribed to tumbling of individual BSA molecules and/or to restricted rotational dynamics within the flexible protein network.

### Generalized relaxation theory

Since  $\tau_2$  is of the same order of magnitude as  $1/\omega_Q$ , the motional-narrowing condition, Eq. 7, is not satisfied. The conventional relaxation theory therefore cannot be used to describe the major dispersion step. Assuming for the moment that all deuterons responsible for the major dispersion step have the same residence time,  $\tau_{\text{res},2}$ , we can use Eqs. 10 and 11 to calculate  $N_2$  and  $\tau_{\text{res},2}$  from the corresponding apparent quantities  $\hat{N}_2$  and  $\hat{\tau}_{\text{res},2}$  given in Table 1. The result of this calculation is presented in Fig. 3 for a range of values of the product  $A\chi$ , corresponding to  $0.47 < A < 0.94$  if the ice  $lh$  value for  $\chi$  is adopted. As expected, both  $N_2$  and  $\tau_{\text{res},2}$  are larger than the corresponding apparent quantities, although the difference is not dramatic within the plausible range of  $A\chi$  values shown.

Although derived with the aid of the generalized relaxation theory, the results in Fig. 3 do not explain the remarkable observation that the inflection frequency of the major dispersion is virtually the same for different (large) proteins and at different temperatures (Koenig et al., 1993b). Furthermore, although the crystal structure of serum albumin has not yet been refined to sufficient resolution to confidently locate water molecules (He and Carter, 1992), the number of buried water molecules in a protein as large as BSA is expected to be an order of magnitude larger than the values implied by Fig. 3 (Rashin et al., 1986; Williams et al., 1986). We are thus led to consider a more realistic model with a wide distribution of residence times for the internal deuterons of BSA.

For simplicity, we assume a rectangular distribution of  $\log(\tau_{\text{res}})$ , corresponding to a uniform distribution of activation energies for the processes (presumably conforma-

tional fluctuations) that limit the rate of escape of buried water molecules into the bulk. Fitting the three parameters in this distributed  $\tau_{\text{res}}$  model to the dispersion data, we obtain  $\alpha = 4 \pm 1 \text{ s}^{-1}$ ,  $N = 49 \pm 4$ , and  $A\chi = 137 \pm 10 \text{ kHz}$  for sample A, and  $\alpha = 4.6 \pm 0.3 \text{ s}^{-1}$ ,  $N = 48 \pm 3$ , and  $A\chi = 190 \pm 10 \text{ kHz}$  for sample B. The fits, shown as solid curves in Fig. 4, are nearly as good as the five-parameter fits of the bimodal model in Fig. 2. In the fits of Fig. 4, the cutoffs for the logarithmically uniform  $\tau_{\text{res}}$  distribution were taken to be  $10^{-9}$  and  $10^{-3} \text{ s}$ . As demonstrated by Fig. 1, however, the dispersion profile is not significantly affected by deuterons with residence times much smaller or much larger than  $1/\omega_Q$ , which equals  $1.55 \mu\text{s}$  (sample A) or  $1.12 \mu\text{s}$  (sample B) according to the fits in Fig. 4. The fits should thus be insensitive to the cutoff values, provided that these are not too close to  $1/\omega_Q$ . This point is illustrated in Fig. 4, showing that the dispersions can be accounted for by 15–20 deuterons with a logarithmically uniform distribution of residence times in the range  $0.1\text{--}10 \mu\text{s}$ . It should be realized, however, that the number of internal deuterons deduced in this way depends to some extent on the assumed shape of the  $\tau_{\text{res}}$  distribution within the range  $0.1\text{--}10 \mu\text{s}$ . Furthermore, BSA may contain several buried water molecules that do not contribute to the dispersion profile since their residence times are outside the  $0.1\text{--}10 \mu\text{s}$  window probed by  $^2\text{H}$  relaxation dispersion profiles from immobilized protein samples. Information about these “invisible” water molecules can be obtained from  $^2\text{H}$  and  $^{17}\text{O}$  relaxation dispersion profiles from solutions of rotationally mobile BSA, where the residence time window is wider, typically  $10^{-8}\text{--}10^{-4} \text{ s}$  (Denisov and Halle, 1995b).

In light of the foregoing analysis, we can readily understand the unexpected invariance of the inflection frequency

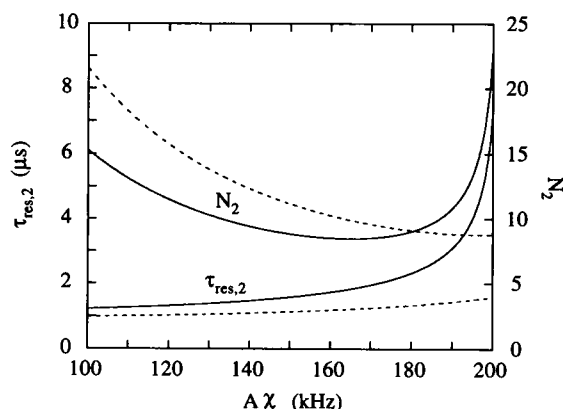


FIGURE 3 The number  $N_2$  of deuterons responsible for the major dispersion step in Fig. 2 and their residence time  $\tau_{\text{res},2}$ , as calculated from the apparent parameters  $\beta_2$  and  $\tau_2$  in Table 1 using the generalized relaxation theory. The solid curves refer to sample A and the dashed curves to sample B.

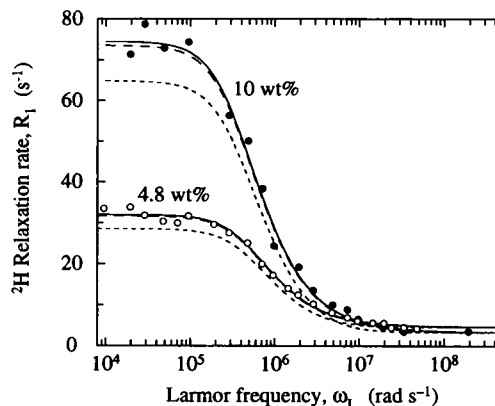


FIGURE 4 Longitudinal relaxation dispersion of water deuterons from chemically cross-linked BSA in sample A (10 wt %, 35°C) (Koenig et al., 1993b) and in sample B (4.8 wt %, 20°C) (Koenig and Brown, 1993). The solid curves resulted from fits of the three parameters  $\alpha$ ,  $N$ , and  $A\chi$ , using the generalized relaxation theory and assuming a logarithmically uniform distribution of residence times in the range  $10^{-9}\text{--}10^{-3} \text{ s}$ . The dashed curves were obtained by shrinking the residence time range to  $10^{-8}\text{--}10^{-4} \text{ s}$  (long dash) or to  $10^{-7}\text{--}10^{-5} \text{ s}$  (short dash), keeping  $\alpha$  and  $A\chi$  fixed but scaling  $N$  to maintain the residence time density. Thus, e.g., the number of deuterons with residence times in the range  $10^{-7}\text{--}10^{-5} \text{ s}$  is 16.4 (sample A) or 16.0 (sample B) for all three curves.

of the dispersion profiles from rotationally immobilized samples made from different proteins and at different temperatures. As long as the residence time distribution spans the critical range around  $1/\omega_Q$ , any such sample will produce a  $^2\text{H}$  dispersion profile with an apparent correlation time near  $1\ \mu\text{s}$ . Similarly, a change of temperature will not affect the dispersion profile much if the number of deuterons with residence times in the range  $0.1\text{--}10\ \mu\text{s}$  is nearly constant. Even if this number varies considerably, the inflection frequency will not be affected.

### Surface waters, buried waters, or labile hydrogens?

In the preceding analysis, we have tacitly assumed that the deuterons responsible for the  $^2\text{H}$  dispersion reside in protein-associated water molecules. It is conceivable, however, that exchanging labile hydrogens in the amino acid side chains of BSA contribute significantly to the  $^2\text{H}$  dispersion. In a recent study of the small globular protein BPTI, we showed that the hydrogen exchange contribution to the  $^2\text{H}$   $R_1$  dispersion is highly pH dependent (Denisov and Halle, 1995b). Only in the neutral pH range 5–7 can hydrogen exchange contributions be neglected in the case of BPTI. At the (nominal) pH value of 7 of samples A and B, nearly all of the 101 carboxyl groups of BSA are deprotonated (Tanford et al., 1955). However, there are 17 histidine residues in BSA, of which roughly half are deprotonated at pH 7 (Tanford et al., 1955; Sadler and Tucker, 1993). Kinetic studies on model compounds show that the acidic imidazole nitrogen of histidine can participate in fast hydrogen exchange (in the  $\mu\text{s}$  range) with protonated amine groups mediated by one or two water molecules (Ralph and Atherton, 1980). Because the amino acid sequence of BSA has eight histidine residues adjacent to lysine or arginine residues (Brown, 1976), this mechanism could well be of importance in BSA in the neutral pH range. (In contrast, BPTI contains no histidine residues.) In conclusion, it appears likely that a significant fraction of the deuterons responsible for the  $^2\text{H}$  dispersion from immobilized BSA are labile side-chain deuterons rather than water deuterons. The labile deuteron contribution should be even more important in solutions of rotationally mobile BSA, where all deuterons exchanging on time scales shorter than the intrinsic  $^2\text{H}$  relaxation rate ( $\sim 10^{-4}\ \text{s}$ ) contribute to the dispersion profile. A quantitative assessment of the labile-deuteron contribution would require a systematic study of the difference in pH dependence between the  $^2\text{H}$  and  $^{17}\text{O}$  relaxation dispersions, as recently carried out for BPTI (Denisov and Halle, 1995a, b).

Although the spin relaxation behavior of the observed bulk water resonance does not directly locate the protein-associated water molecules responsible for the relaxation dispersion, recent extensive  $^{17}\text{O}$  and  $^2\text{H}$  relaxation dispersion studies of several globular proteins (Denisov and Halle, 1995a, b; Denisov and Halle, in preparation), including

mutant forms with displaced internal water molecules (V. Denisov, submitted for publication), have unambiguously identified the buried water molecules seen in high-resolution crystal structures as the source of the relaxation dispersion. Although the crystal structure of serum albumin has not yet been refined sufficiently to identify water molecules (He and Carter, 1992), an analysis of 75 high-resolution crystal structures of monomeric proteins yields an average of one buried water molecule per 27 residues (Williams et al., 1986). For the 581 residues of BSA, this translates into 22 buried water molecules. In addition, water molecules may be trapped in the inter-subdomain regions of the BSA molecule (He and Carter, 1992). Such water molecules should be released at low pH, where the three subdomains separate (Peters, 1975). The low-frequency component of the  $^{17}\text{O}$  relaxation rate is, indeed, drastically reduced at low pH (Halle et al., 1981).

As we have previously suggested (Denisov and Halle, 1995a), buried water molecules owe their long residence times (compared with bulk water) not to a full complement of strong hydrogen bonds to the protein, but rather to the free energy cost of creating a transient, locally unfolded protein conformation or to the need for a buried water molecule to surmount free energy barriers associated with poorly hydrogen-bonded intermediate states on its way out of a pore or narrow cleft. Other authors have ascribed long water residence times to strong hydrogen bonds with the protein surface (Koenig et al., 1993b; Iino, 1994), or to a protein-induced modification of the water structure (Kimmich et al., 1990). Although many water molecules at the protein surface are undoubtedly hydrogen bonded to protein atoms (Baker and Hubbard, 1984; Thanki et al., 1988), unless they reside in pores or narrow clefts, these hydrogen bonds can presumably be broken in a similar cooperative manner as in bulk water and, hence, should not give rise to very long residence times. The accumulated evidence from computer simulations (Daggett and Levitt, 1993; Brunne et al., 1993), high-resolution multidimensional  $^1\text{H}$  NMR spectroscopy (Otting et al., 1991), and  $^{17}\text{O}$  relaxation dispersion (Denisov and Halle, 1995a, b) unanimously support a picture of highly mobile (sub-nanosecond) water molecules at the surface of globular proteins.

### CONCLUSIONS

We have shown here that the apparent correlation time of  $1\ \mu\text{s}$ , deduced from  $^2\text{H}$  relaxation dispersion profiles from several immobilized proteins at different temperatures (Kimmich et al., 1990; Koenig et al., 1993b; Koenig and Brown, 1993), does not reflect a universal dynamic behavior of protein-associated water molecules, as previously suggested (Koenig et al., 1993a; Koenig and Brown, 1993, 1994). Using a stochastic theory of spin relaxation (B. Halle, submitted for publication), which, in contrast to the conventional perturbation theory, is valid for slowly exchanging quadrupolar nuclei in immobilized protein sam-

ples, we have demonstrated that the apparent correlation time of 1  $\mu$ s is nothing but the inverse of the deuteron quadrupole frequency, which indeed is expected to be independent of temperature and protein structure. Any residence time distribution spanning the  $\mu$ s range gives rise to a dispersion profile with an apparent correlation time close to the inverse quadrupole frequency. Adopting a logarithmically uniform distribution, we showed that the observed  $^2$ H dispersion can be accounted for by 15–20 deuterons with residence times in the range 0.1–10  $\mu$ s. If the actual distribution is peaked near 1  $\mu$ s, this number would be smaller, and vice versa. While a significant contribution from labile protein deuterons cannot be ruled out, we argue that the water molecules that contribute to the dispersion profile are buried within the protein, rather than located at its surface. This view harmonizes with our recent results for BPTI, where three of the four buried water molecules have residence times in the range 15 ns–1  $\mu$ s and the remaining one in the range 10–200  $\mu$ s (Denisov and Halle, 1995a, b; V. Denisov, submitted for publication). Given that the  $^2$ H dispersion from immobilized protein samples is significantly affected only by deuterons with residence times in the range 0.1–10  $\mu$ s, it should reveal only a subset of the expected 20 or more buried water molecules in BSA. Finally, we note that the present results have implications also for the interpretation of  $^1$ H relaxation data from immobilized protein samples (Grösch and Noack, 1976; Oakes, 1976; Conti, 1986; Hills et al., 1989; Koenig and Brown, 1991, 1993, 1994; Brown and Koenig, 1992; Hills, 1992; Koenig et al., 1993a, b; Iino, 1994; Hinton and Bryant, 1994; Zhou and Bryant, 1994) and for magnetic resonance imaging of soft tissue.

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