Hydrogen Exchange and Protein Hydration: The Deuteron Spin Relaxation Dispersions of Bovine Pancreatic Trypsin Inhibitor and Ubiquitin

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Water deuteron (2H) spin relaxation was used to study hydrogen exchange, hydration, and protein dynamics in aqueous solutions of the globular proteins bovine pancreatic trypsin inhibitor (BPTI) and ubiquitin. The frequency dispersion of the longitudinal 2H relaxation rate was measured in the pD range 2 to 11 at 27°C. In contrast to the previously reported water 17O relaxation dispersion from the same samples, the 2H dispersion depends strongly on pD. This pD dependence is due to labile protein deuterons in acidic side-chains and surface peptide groups, which exchange rapidly with water deuterons. The pD dependence of the 2H relaxation in BPTI solutions could be quantitatively accounted for in terms of known pK values and hydrogen exchange rate constants. For ubiquitin, labile protein deuterons contribute importantly to the 2H relaxation dispersion even in the neutral pD range. The 2H relaxation data also provided information about the orientational order and internal motion of OD and ND bonds in side-chains and surface peptides. A comparison of the water contribution to the 2H dispersion with the 17O dispersion indicates that one of the four internal water molecules of BPTI, presumably the deeply buried water molecule W122, exchanges more slowly (10⁻⁶ to 10⁻⁴ second) than the other three (10⁻⁸ to 10⁻⁶ second).

Keywords: hydrogen exchange; protein hydration; nuclear magnetic resonance; bovine pancreatic trypsin inhibitor; ubiquitin

Introduction

The nuclear spin relaxation dispersion of the hydrogen isotopes 1H and 2H in aqueous protein solutions has been used extensively during the past three decades to characterize the water-protein interaction, in particular, its dynamic aspects (Daszkiewicz et al., 1963; Koenig & Schillinger, 1969; Kimmich & Noack, 1971; Koenig et al., 1975; Hallenga & Koenig, 1976; Grösch & Noack, 1976; Koenig, 1980; Shirley & Bryant, 1982; Bryant & Jarvis, 1984; Conti, 1986; Bryant, 1988; Schauer et al., 1988; Hills et al., 1989; Kimmich et al., 1990; Prosser & Peemöller, 1991; Koenig & Brown, 1991; Hills, 1992; Koenig et al., 1993; Hills & Favret, 1994; Belton, 1994). A potential complication in such studies is the exchange of hydrogen nuclei between water and protein. Although discussed at an early stage (Koenig & Schillinger, 1969), the effect of hydrogen exchange on spin relaxation has been dismissed as unimportant in most subsequent work. A quantitative justification of this point of view, however, has not been presented.

All globular proteins contain large numbers of labile hydrogen atoms that can exchange with the hydrogen atoms of the aqueous solvent. Even in a small protein like bovine pancreatic trypsin inhibitor (BPTI) there are, depending on pH, between 103 and 119 labile hydrogen atoms. The exchange rates of labile hydrogen atoms in globular proteins span some 15 orders of magnitude, with residence times (the inverse of the exchange rates) ranging from submicrosecond for solvent-accessible carboxyl groups to years for deeply buried water molecules. Whereas most studies of hydrogen exchange kinetics in globular proteins have focused on the internal peptide amide protons, with residence times of order ten minutes or longer (Wagner, 1983), it is only the solvent-accessible amide protons (Tüchsen & Woodward, 1985) and the labile side-chain hydrogen atoms in carboxyl (Lankhorst et al., 1983), hydroxyl (Liepinsh et al., 1992), ammonium, imidazolium, and...
guanidinium (Eigen, 1964) groups that exchange sufficiently rapidly to contribute to the spin relaxation of the water $^1$H or $^2$H resonance.

Water-protein hydrogen exchange can effect the spin relaxation behaviour of the observed water $^1$H or $^2$H resonance by two distinct mechanisms, involving the differences in intrinsic spin relaxation rates and chemical shifts between protein and water hydrogen atoms. In the first case, the rapidly relaxing protein hydrogen atoms act as relaxation sinks for the much more slowly relaxing water hydrogen atoms, provided that the hydrogen exchange is fast compared to the relaxation rate difference so that magnetization loss by exchange can compete with magnetization loss by relaxation (Piculell & Halle, 1986; Hills, 1992; Belton, 1994). In the second case, hydrogen exchange renders the chemical shielding of the $^1$H or $^2$H nucleus time-dependent, thereby inducing spin relaxation or loss of coherence (Hills et al., 1989; Belton, 1994). Since the exchange rate is usually much smaller than the Larmor frequency, this mechanism only affects the transverse spin relaxation rate.

In the preceding paper (Denisov & Halle, 1995), we reported the $^{17}$O relaxation dispersions from the same protein solutions of the two small globular proteins BPTI and ubiquitin. Being unaffected by hydrogen exchange with the protein, the $^{17}$O relaxation reports exclusively on the behaviour of the water molecules in a protein solution (Halle et al., 1981; Piculell & Halle, 1986). A major conclusion of the preceding $^{17}$O study was that the water molecules at the protein surface are highly mobile and do not contribute to the observed relaxation dispersion, which is entirely due to a few highly ordered water molecules completely or partly buried within the protein. Here we report the $^2$H relaxation dispersions from the same protein solutions. By comparing the $^1$H and $^{17}$O dispersions at eight different pD values in the range 2 to 11, we can separate the contributions to the longitudinal $^1$H relaxation rate from labile protein deuterons and from water deuterons. For BPTI, where most of the relevant pK values and exchange rate constants are known, the labile deuteron contribution can be shown to have the expected pD dependence. While, for BPTI, there exists a narrow pD range where labile protein deuterons do not contribute significantly to the longitudinal $^1$H relaxation, this contribution is at least as large as the water contribution for ubiquitin.

In general, the residence time $\tau_{res}$ of a solvent-accessible labile deuteron in a globular protein can be expressed as:

$$\frac{1}{\tau_{res}} = k_0^D + k_0^A[D_2O^+] + k_0^{OD^-},$$

(1)

with $k_0^D$ and $k_0^A$ the second-order rate constants for the acid and base-catalyzed exchange mechanisms, and $k_0^{OD^-}$ the first-order rate constant for uncatalyzed exchange. Whereas rate constants for hydrogen exchange in a variety of small molecules have been reported (Grunwald et al., 1957; Loewenstein & Meiboom, 1957; Berger et al., 1959; Luz & Meiboom, 1963; Eigen, 1964; Lankhorst et al., 1983; Hills, 1992), little information is available for globular proteins. In the case of BPTI, however, the rate constants for the solvent-accessible peptide amide groups (Tüchsen & Woodward, 1985) and for the hydroxyl groups of serine, threonine, and tyrosine (Liepinsh et al., 1992) have been determined. The carboxyl hydrogen is believed to exchange predominantly via an uncatalyzed mechanism involving two water molecules in a hydrogen-bonded hexagonal ring (Luz & Meiboom, 1963). The pD-independent deuteron residence time in acetic acid dissolved in $^2$H$_2$O is 0.36 microsecond at 25°C (Lankhorst et al., 1983). Deuterons in the solvent-accessible carboxyl groups of globular proteins should thus be in the fast-exchange limit (see below) at any pD value. The three ammonium hydrogen atoms of lysine (and the N terminus) and the four guanidinium hydrogen atoms of arginine are predominantly exchanged via the base-catalyzed mechanism. In small molecular mass model compounds the rate constant is close to the diffusion limit (Eigen, 1964). An analysis of our relaxation data (see Hydrogen exchange: pD dependence of $t_{res}$) yields an average rate constant of $k_0^{OD^-} = 6.1 \times 10^7$ M$^{-1}$ s$^{-1}$ for the lysine and arginine residues of BPTI, a factor 3 below the diffusion limit.

For BPTI, the acid dissociation constants of all titrating residues have been determined (Wüthrich & Wagner, 1979). These are collected, together with all relevant deuteron exchange rate constants, in Tables 1 and 2. (We assume that the $\epsilon$-ND deuteron of arginine and the primary amide deuterons of glutamine and asparagine exchange slowly on the $^2$H relaxation time-scale).

A given labile deuteron contributes to the observed $^2$H relaxation only if its residence time $\tau_{res}$ is shorter than, or comparable to, its intrinsic longitudinal spin relaxation time $1/R$ (see Deuteron spin relaxation in protein solutions), which is typically in the range one to ten milliseconds (depending on the Larmor frequency). The effective number, $N_{\tau}$, of labile
deuterons that contribute to the $^2$H relaxation at a given pD value can thus be roughly estimated as:

$$N_0 = \sum \frac{N_i}{1 + 10^{pD - pH_i}}$$  \hspace{1cm} (2)

where the sum runs over all amino acid residues, each containing $N_i$ labile deuterons at a given pD value:

$$N_i = \frac{N^0_i}{1 + 10^{pD - pH_i}}$$  \hspace{1cm} (3)

Here $N^0_i$ is the number of labile deuterons in the acidic form of the residue (at pD $\ll pK_i$), $R_i$ is the intrinsic relaxation rate of the labile deuteron, and $t_{100}^i$ is its residence time, as given by equation (1) with $[D_3O^+]_i = 10^{-pD}$, $[OD_i] = 10^{pD - pK_i}$, and $pK_W = 14.80$ in $^2$H$_2$O at 27°C (Covington et al., 1966). Using pK values and rate constants from Tables 1 and 2, and the intrinsic relaxation rates deduced from our $^2$H relaxation data (see Hydrogen exchange: pD dependence of $x$ and $\beta$ below), we have calculated the effective number, $N_D$, of labile BPTI deuterons that contribute at a given pD to the $^2$H relaxation at high ($x$) and low ($\beta$) frequency, as well as the contributions to $N_D$ from different types of labile deuterons. As seen from Figure 1, the five carboxyl and seven of the eight hydroxyl deuterons are in fast exchange at pD 2, while seven of the eight hydroxyl, 10 to 12 of the 12 lysine, the 24 arginine, and 5 to 15 of the 53 peptide amide deuterons are in fast exchange at pD 2, while seven of the eight hydroxyl, 10 to 12 of the 12 lysine, the 24 arginine, and 5 to 15 of the 53 peptide amide deuterons are in fast exchange at pD 10. In the neutral pD range only a few of the labile deuterons in BPTI are expected to contribute to the $^2$H relaxation.

### Deuteron spin relaxation in protein solutions

The longitudinal relaxation rate $R_i$ of the quadrupolar $^2$H nucleus in an aqueous protein solution can be expressed as (Piculell & Halle, 1986):

$$R_i(\omega_b) = R_{\text{bulk}} + \frac{2}{1 + \omega_b \tau_c} + \frac{0.8}{1 + (2\omega_b \tau_c)^2},$$  \hspace{1cm} (4)

\[ \text{JMB—MS 298} \]

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Residue(s)</th>
<th>pK**</th>
<th>Rate constants$^a$</th>
</tr>
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<tr>
<td>COOD</td>
<td>Ala58 (C terminus)</td>
<td>3.3</td>
<td>$k_i^b \approx 2.4 \times 10^{10}$</td>
</tr>
<tr>
<td>COOD</td>
<td>Asp3 and 50</td>
<td>3.8; 3.4</td>
<td>$k_i^b$</td>
</tr>
<tr>
<td>COOD</td>
<td>Glu7 and 49</td>
<td>4.1; 4.2</td>
<td>$k_i^b$</td>
</tr>
<tr>
<td>OD</td>
<td>Ser47</td>
<td>c</td>
<td>$k_i^b$ = 7.5 $\times$ 10$^6$</td>
</tr>
<tr>
<td>OD</td>
<td>Thr11</td>
<td>c</td>
<td>$k_i^b$ = 1.8 $\times$ 10$^6$</td>
</tr>
<tr>
<td>OD</td>
<td>Thr32</td>
<td>c</td>
<td>$k_i^b$ = 2.0 $\times$ 10$^6$</td>
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<tr>
<td>OD</td>
<td>Thr54</td>
<td>c</td>
<td>$k_i^b$ = 4.8 $\times$ 10$^6$</td>
</tr>
<tr>
<td>OD</td>
<td>Tyr10</td>
<td>10.3</td>
<td>$k_i^b$</td>
</tr>
<tr>
<td>OD</td>
<td>Tyr21</td>
<td>10.7</td>
<td>$k_i^b$ = 2.6 $\times$ 10$^6$</td>
</tr>
<tr>
<td>OD</td>
<td>Tyr23</td>
<td>11.9</td>
<td>$k_i^b$</td>
</tr>
<tr>
<td>OD</td>
<td>Tyr25</td>
<td>11.5</td>
<td>$k_i^b$</td>
</tr>
<tr>
<td>ND*</td>
<td>Arg1 (N terminus)</td>
<td>8.5</td>
<td>$k_i^b$</td>
</tr>
<tr>
<td>ND*</td>
<td>Lys15, 26 and 46</td>
<td>11.0</td>
<td>$k_i^b$ = 6.1 $\times$ 10$^4$</td>
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<tr>
<td>ND*</td>
<td>Lys41</td>
<td>11.2</td>
<td>$k_i^b$</td>
</tr>
<tr>
<td>(ND$^2$)*</td>
<td>Arg1, 17, 20, 39, 42, 52</td>
<td>c</td>
<td>$k_i^b$</td>
</tr>
</tbody>
</table>

$^a$ pK** values in $^2$H$_2$O at 35°C from Wüthrich & Wagner (1979), converted to the standardized pD scale (Covington et al., 1968) according to $pK_0^* = pK^* + 0.41$.

$^b$ Rate constants in units of s$^{-1}$ ($k_i^b$) or M$^{-1}$ s$^{-1}$ ($k_i^b$ and $k_i^b$).

$^c$ Assumed to be in the acidic form at pD $<$ 11.

$^d$ For acetic acid in $^2$H$_2$O at 25°C (Lankhorst et al., 1983).

$^e$ Rate constants, determined by Liepinsh et al. (1992) in 90% H$_2$O/10% $^2$H$_2$O at 4°C, have been converted to $^2$H$_2$O at 27°C assuming $k \approx T/\eta$ and a primary $^2$H/$^2$H isotope effect of 2. The unresolved residues Tyr10 and Tyr21 are given the same rate constants as Tyr23. For the intramolecularly hydrogen-bonded Tyr35, the similarly corrected rate law is (Liepinsh et al., 1992):

$$\frac{1}{t_{100}} = 0.020 \times 10^{0.07pD} + 41 \times 10^{-0.4pD}.$$

$^f$ Estimated from the $^1$H relaxation data of the present work.

### Table 2

<table>
<thead>
<tr>
<th>Residue</th>
<th>$k_i^b$ (M$^{-1}$ s$^{-1}$)</th>
<th>Residue</th>
<th>$k_i^b$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
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<tr>
<td>Asp3</td>
<td>5.5 $\times$ 10$^6$</td>
<td>Thr11</td>
<td>3.6 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Asp50</td>
<td>2.3 $\times$ 10$^6$</td>
<td>Ala40</td>
<td>2.9 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Lys46</td>
<td>7.8 $\times$ 10$^6$</td>
<td>Arg17</td>
<td>1.9 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Arg39</td>
<td>3.5 $\times$ 10$^6$</td>
<td>Gly57</td>
<td>1.2 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Glu49</td>
<td>2.8 $\times$ 10$^6$</td>
<td>Ala25</td>
<td>4.2 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Phe4</td>
<td>1.8 $\times$ 10$^6$</td>
<td>Cys30</td>
<td>1.8 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Ser47</td>
<td>1.1 $\times$ 10$^6$</td>
<td>Gly12</td>
<td>1.2 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Arg42</td>
<td>1.0 $\times$ 10$^6$</td>
<td>Ala27</td>
<td>8.4 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Lys15</td>
<td>6.2 $\times$ 10$^6$</td>
<td>Ile19</td>
<td>5.5 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Lys26</td>
<td>4.5 $\times$ 10$^6$</td>
<td>Val34</td>
<td>4.2 $\times$ 10$^6$</td>
</tr>
<tr>
<td>Ala48</td>
<td>4.2 $\times$ 10$^6$</td>
<td>Thr32</td>
<td>3.0 $\times$ 10$^6$</td>
</tr>
</tbody>
</table>

The rate constants, determined by Tüchsen & Woodward (1985), have been multiplied by a factor $10^{0.41}$ to convert to the standardized pD scale and by another factor 0.5 to approximately correct for the $^1$H/$^2$H isotope effect.
Hydrogen Exchange and Protein Hydration

Figure 1. The effective number \( N_D \) of labile BPTI deuterons expected to contribute to the longitudinal \(^2\text{H}\) spin relaxation rate of the water resonance at (a) high frequency (\( \alpha \) parameter), and (b) low frequency (\( \beta \) parameter).

\[
\alpha = f_s(\langle R_S \rangle - R_{\text{bulk}}), \quad (6a)
\]
\[
\beta = \frac{3\pi^2}{2} f_i \langle A_i^2 \rangle, \quad (6b)
\]

where \( f_s \) and \( f_i \) are the fractions of all deuterons contributing to the \(^2\text{H}\) resonance that belong to water molecules on the surface of the protein and to internal (buried) water molecules, respectively. Furthermore, \( \langle R_S \rangle \) is the average intrinsic \(^2\text{H}\) relaxation rate of surface water, and \( A_i \) and \( j_i \) are the generalized order parameter and quadrupole coupling constant for \(^2\text{H}\) in internal water molecules. The expressions (6) differ from the corresponding ones for \(^1\text{H}\) relaxation (Denisov & Halle, 1995) only in the spin-dependent numerical factor in equation (6b). \(^2\text{H}\) has spin quantum number \( I = 1 \), while \(^1\text{H}\) has \( I = 5/2 \).

The contributions \( \alpha_P \) and \( \beta_P \) from the labile protein deuterons can be expressed as sums over all exchanging deuterons:

\[
\alpha_P = \sum_i f_i \alpha_i, \quad (7a)
\]
\[
\beta_P = \sum_i f_i \beta_i, \quad (7b)
\]

where \( \tau_8 \) is the rotational correlation time of the protein. To obtain equation (7) we have expressed the zero-frequency intrinsic longitudinal \(^2\text{H}\) relaxation rate of a labile protein deuteron as

\[
\alpha_i = \frac{f_i \alpha_i}{1 + \tau_{8i} \tau_{a}}, \quad (5a)
\]
\[
\beta_i = \frac{f_i \beta_i}{1 + \tau_{8i} \tau_{a}}, \quad (5b)
\]

with \( \tau_{8i} \) the known bulk water \(^2\text{H}\) relaxation rate (see Materials and Methods, Relaxation dispersion measurements) and \( \tau_{a} \), the correlation time characterizing the relaxation dispersion. In contrast to the case of \(^1\text{O}\) relaxation (Denisov & Halle, 1995), where the parameters \( \alpha \) and \( \beta \) are due exclusively to water molecules, the corresponding \(^2\text{H}\) parameters in equation (4) in general contain contributions from deuterons in water molecules as well as from the labile deuterons of the protein. These parameters can be decomposed as:

\[
\alpha = \alpha_W + \alpha_P, \quad (5a)
\]
\[
\beta = \beta_W + \beta_P, \quad (5b)
\]

where \( \alpha_W \) and \( \beta_W \) are due to surface and internal water molecules, respectively. These quantities can be expressed as (Piculell & Halle, 1986; Denisov & Halle, 1995):

\[
\alpha_W = \frac{N_W}{2N_W + \sum_i N_i}, \quad (8)
\]

where \( N_W \) is the total number of water molecules per protein molecule in the solution and \( N_i \) is given by equation (3).

Relaxation dispersion in BPTI and ubiquitin solutions

The \(^2\text{H}\) relaxation dispersions in solutions of BPTI and ubiquitin, each at two different pD values, are shown in Figure 2. The parameters \( \alpha \), \( \beta \), and \( \tau_8 \) in equation (4) were determined from non-linear least-squares fits to these data and to similar data obtained at other pD values in the range 2 to 11. The results of the \(^2\text{H}\) relaxation dispersion fits at all eight investigated pD values are shown as three-dimensional plots in Figures 3 and 4, which also include the
Figure 2. Deuteron longitudinal relaxation dispersion in solutions of BPTI (●, 10.4% (w/w)) and ubiquitin (○, 10.2% (w/w)) in 2H2O at 27°C, each at 2 pD values. The continuous curves resulted from fits of the parameters $\alpha$, $\beta$, and $\tau$ in equation (4).

Figure 3. Longitudinal relaxation rate, $R_1$, for water $^1$H (top) and $^{17}$O (bottom) in BPTI solutions (10.4% (w/w), 2H2O, 27°C) versus Larmor frequency $\omega_0/(2\pi)$, and pD. The continuous curves on the $R_1$ surfaces were obtained from fits (like those in Figure 2) to relaxation dispersion data measured at pD 1.9, 3.4, 4.7, 5.6, 7.0, 8.3, 10.0, and 10.7. The unshaded plane corresponds to $R_1$ for bulk water.

Figure 4. Longitudinal relaxation rate, $R_1$, for water $^2$H (top) and $^{17}$O (bottom) in ubiquitin solutions (10.2% (w/w), 2H2O, 27°C) versus Larmor frequency $\omega_0/(2\pi)$, and pD. The continuous curves on the $R_1$ surfaces were obtained from fits (like those in Figure 2) to relaxation dispersion data measured at pD 1.9, 3.4, 4.6, 5.8, 7.4, 9.0, and 10.5. The unshaded plane corresponds to $R_1$ for bulk water.

corresponding $^{17}$O results (Denisov & Halle, 1995). Comparing these $^1$H and $^{17}$O dispersions, measured on the same samples, we note two qualitative differences. First, unlike the $^{17}$O dispersion, the $^1$H dispersion is strongly pD-dependent. Second, while the $^{17}$O dispersion step for ubiquitin is less than 10% of $R_{bulk}$ at all pD values, the $^1$H dispersion step is comparable to or larger than $R_{bulk}$ (depending on pD). In Hydrogen exchange: pD dependence of $\alpha$ and $\beta$, below, we show that this difference can be quantitatively accounted for in terms of the exchanging protein deuterons.

We have also measured the dispersion of the transverse $^1$H relaxation rate $R_2$ in all our samples. Unlike $R_1$, which is entirely due to quadrupolar relaxation, $R_2$ also contains contributions from scalar relaxation of the first kind (Abragam, 1961) and, at the higher fields, from chemical shift modulation (Hills et al., 1989). The scalar relaxation contribution, due to the modulation of the $^1$H-$^{17}$O indirect spin coupling by deuteron exchange in water molecules, is, as expected, independent of Larmor frequency and shows the characteristic maximum near pD 7 (Meiboom, 1961; Halle & Karlström, 1983). At pD 1.9, where the acid-catalyzed deuteron exchange in water is sufficiently fast to eliminate the scalar relaxation contribution, $R_2$ exceeds $R_1$ by less than 5%. This observation ensures that the levelling off of $R_2$ seen in Figures 2 to 4 represents the true zero-frequency plateau.

The second non-quadrupolar $R_2$ contribution is due to the difference in chemical shift between labile protein deuterons and water deuterons, which can be as large as 5 p.p.m. (Wüthrich, 1986). As the deuterons exchange between protein and water their chemical shielding fluctuates in time, thus contributing to the dephasing of the transverse magnetization. Since the absolute chemical shift is proportional to
Figure 5. pD dependence of the correlation time $\tau_c$, determined independently from the $^2$H and $^{17}$O relaxation dispersions in the same BPTI solution (10.4% (w/w), $^2$H$_2$O, 27°C).

Figure 6. pD dependence of the correlation time $\tau_c$, determined from the $^2$H relaxation dispersion in ubiquitin solution (10.2% (w/w), $^2$H$_2$O, 27°C).

Hydrogen exchange: pD dependence of $\alpha$ and $\beta$

Figures 7 and 8 show the variation with pD of the parameters $\alpha$ and $\beta$, derived from the $^2$H relaxation dispersions of BPTI and ubiquitin solutions shown in Figures 2 to 4. In contrast to the corresponding $^{17}$O parameters, which show very little pD dependence (Denisov & Halle, 1995), the $^2$H parameters vary strongly with pD. A comparison with Figure 1 clearly shows that this pD dependence is due to exchanging protein deuterons, and, hence, should conform to equations (5) and (7), with the pD dependence entering via $\tau_{Pi}$ and $f_{Pi}$ as given by equations (1), (3), and (8). In the case of BPTI, these two quantities can be calculated as functions of pD from the $pK$ values and rate constants given in Tables 1 and 2.

To quantitatively account for the data in Figure 7, we must also specify the intrinsic relaxation parameters $\alpha_i$ and $\beta_i$ (assumed to be independent of pD) for all labile BPTI deuterons. For simplicity, we divide the labile deuterons into three classes: deuterons bound to oxygen atoms in carboxyl and hydroxyl groups (OD); deuterons bound to nitrogen atoms in the side-chains of lysine and arginine (NDs); and deuterons bound to nitrogen atoms in the main-chain peptide groups (NDp). We thus have six relaxation parameters: $\alpha_{OD}$, $\alpha_{ODr}$, $\alpha_{ODp}$, $\beta_{OD}$, $\beta_{ODr}$, and $\beta_{ODp}$. Rather than regarding the water contributions $\alpha_w$ and $\beta_w$ as constants, we assume that they are proportional to the previously determined (Denisov & Halle, 1995) $\alpha(^{17}$O) and $\beta(^{17}$O), respectively, thereby taking into account the weak pD dependence of the water contributions. These relaxation par-
ameters, as well as the unknown rate constant $k^2_{\text{NDs}}$ for the base-catalyzed exchange of labile deuterons in lysine and arginine side-chains, were determined from a non-linear least-squares fit to the combined $\alpha$ and $\beta$ data. The excellent fits, shown in Figure 7, demonstrate that the pD dependence of the $^2$H relaxation can be accounted for quantitatively in terms of labile protein deuterons. We emphasize that the excellent agreement is not simply a consequence of the large number of parameters. As is evident from Figure 1, the qualitative features of the pD dependence are determined by the fixed pK values and rate constants. The relaxation parameters $\alpha_w$ and $\beta_w$ essentially determine the magnitude of the steps in $\alpha$ and $\beta$ at low and high pD values. Thus, for example, $\beta_{\text{OD}}$ can be estimated directly from the low-pD step in Figure 7(b) as (see equations (7b) and (8)):

$$\beta_{\text{OD}} \approx \frac{2N_A}{N_{\text{OD}}} \left[ \beta_{\text{pD 1.92}} - \beta_{\text{pD 5.55}} \right]$$

$$\approx \frac{5210}{12} (1.14 - 0.70) \times 10^9 = 1.9 \times 10^{11} \text{ s}^{-2}. \quad (9)$$

The difference from the value $\beta_{\text{OD}} = 1.5 \times 10^{11} \text{ s}^{-2}$ resulting from the fit is due to the slight pD dependence of $\beta_w$.

Further support for ascribing the pD dependence of the $^2$H relaxation to labile protein deuterons comes from an examination of the parameter values resulting from the fit (see Table 3). As expected (Eigen, 1964), $k^2_{\text{NDs}}$ is close to the diffusion limit, the factor of 3 reduction being ascribable to steric
restrictions and intraprotein hydrogen bonding of some lysine and arginine residues. The intrinsic relaxation parameters can be expressed as (Halle & Wennström, 1981):

\begin{align}
\alpha_{\text{Pi}} &= \frac{3\pi^2}{2} (1 - A_{\text{Pi}}^2) - \chi_{\text{Pi}}^2 \tau_{\text{Pi}}, \\
\beta_{\text{Pi}} &= \frac{3\pi^2}{2} A_{\text{Pi}}^2 \chi_{\text{Pi}}^2
\end{align}

where \( \tau_{\text{Pi}} \) is the effective correlation time for internal motion (restricted rotation) of the OD or ND bond. The \(^2\)H quadrupole coupling constants \( \chi_{\text{Pi}} \) can be estimated from nuclear quadrupole resonance data on crystalline amino acids (Hunt & Mackay, 1974; Edmonds, 1977): \( \chi_{\text{OD}} = 200(\pm 20) \text{ kHz} \), \( \chi_{\text{ND}} = 170(\pm 10) \text{ kHz} \), and \( \chi_{\text{NDP}} = 195(\pm 10) \text{ kHz} \). Using equation (10) and parameter values from Table 3, we thus obtain for the generalized order parameters: \( A_{\text{OD}} = 0.50(\pm 0.05) \), \( A_{\text{ND}} = 0.55(\pm 0.04) \), and \( A_{\text{NDP}} = 0.85(\pm 0.06) \), and for the effective correlation times: \( \tau_{\text{OD}} = 0.12(\pm 0.05) \text{ ns} \), \( \tau_{\text{ND}} = 0.31(\pm 0.06) \text{ ns} \), and \( \tau_{\text{NDP}} = 1.1(\pm 0.3) \text{ ns} \). The average order parameter \( A_{\text{NDP}} = 0.85 \) for the peptide ND bonds closely agrees with the value 0.9 typically found from \(^2\)N relaxation on small globular proteins (Schneider et al., 1992; Stone et al., 1992; Peng & Wagner, 1992; Akke et al., 1993). The side-chain order parameters are smaller, as expected from their internal degrees of freedom. The effective correlation time \( \tau_{\text{NDP}} \), however, is considerably longer than that derived from \(^2\)N relaxation at a single field.

The ubiquitin data in Figure 8 cannot at present be analyzed quantitatively, since the required pK values and rate constants are not available. However, assuming that the ratios \( \alpha_{\text{Pi}}/\alpha_{\text{Pi}}(\text{H}) \) and \( \beta_{\Pi}(\text{H})/\beta_{\Pi}^2(\text{H}) \) are the same as for BPTI (see Protein hydration: comparison of \(^2\)H and \(^1\)O data, below), we can obtain the water contributions \( \alpha_{\text{Pi}} \) and \( \beta_{\Pi} \) from the \(^1\)O data for ubiquitin (Denisov & Halle, 1995). The labile deuteron contributions \( \alpha_{\Pi} \) and \( \beta_{\Pi} \), i.e. the difference between the filled and open points in Figure 8, display qualitatively the same pD dependence as for BPTI, although the low-pD step is larger. This difference is due to the larger number of carboxyl (12) and hydroxyl (11) groups in ubiquitin as compared to BPTI (five and eight). This assertion can be quantified by calculating \( \beta_{\Pi} \) from the ubiquitin data at the lowest pD value in Figure 8(b) and showing that it agrees with the \( \beta_{\Pi} \) value obtained for BPTI. In analogy with equation (9):

\[
\beta_{\Pi} = \frac{2N_{\text{H}}}{N_{\text{OD}}} [\beta(\text{pD} 1.89) - \beta_{\Pi}(\text{pD} 1.89)]
\]

\[
\approx \frac{7010}{23} (0.55 - 0.05) \times 10^9 = 1.5 \times 10^{11} \text{ s}^{-2}, \quad (11)
\]

Table 3

<table>
<thead>
<tr>
<th>Parameters derived from the pD dependence of the (^2)H and (^1)O relaxation dispersions in BPTI solutions</th>
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<tbody>
<tr>
<td>( \alpha_{\text{OD}}(\text{s}^{-1}) = 50 \pm 20 )</td>
</tr>
<tr>
<td>( \alpha_{\text{ND}}(\text{s}^{-1}) = 90 \pm 10 )</td>
</tr>
<tr>
<td>( \alpha_{\text{NDP}}(\text{s}^{-1}) = 180 \pm 30 )</td>
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\( \alpha(\text{H})/\alpha(\text{H}) = 83 \pm 3 \)  
\( k_{\text{2H}}(\text{H}) = (6.1 \pm 0.7) \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \)

where \( \alpha(\text{H})/\alpha(\text{H}) \) can be obtained for BPTI. In analogy with equation (9):
we thus obtain $1/R_i \approx 4 \mu s$ for $^{17}$O and 0.2 ms for $^2$H. A highly ordered internal water molecule with a residence time between these values should contribute to $^2$H but not to $^{17}$O relaxation. The quantities $\beta_w(^{17}$O) and $\beta(^2$H) are therefore not necessarily due to the same number of internal water molecules and their ratio must be written:

$$\frac{\beta(^{17}$O)}{\beta_w(^2$H)} = \frac{8}{125} \frac{N_i(^{17}$O)}{N_i(^2$H)} \frac{\langle \gamma_i(^{17}$O) A_i(^{17}$O) \rangle}{\langle \gamma_i(^2$H) A_i(^2$H) \rangle}. \quad (13)$$

Although the individual quadrupole coupling constants vary considerably with the OD bond length in the water molecule ($^2$H) and with hydrogen-bond induced electronic polarization ($^{17}$O), their ratio is remarkably constant, falling in the narrow range $\chi(^{17}$O)/$\chi(^2$H) = 30.5(±1.5) for a variety of crystal hydrates and for the different ice polymorphs (Poplett, 1982). Using our experimental value $\beta(^{17}$O)/$\beta_w(^2$H) = 46(±1), deduced from the BPTI data (see Table 3), we thus obtain with equation (13) $N_i(^2$H)/$A_i(^2$H) = (1.3(±0.1))$N_i(^{17}$O)/$A_i(^{17}$O). With $N_i(^{17}$O)/$A_i(^{17}$O) = 2.1(±0.1), as deduced from the $^{17}$O relaxation for BPTI in the neutral pH range (Denisov & Halle, 1995), we obtain $N_i(^2$H)/$A_i(^2$H) = 1.3$\times$2.1 = 2.7(±0.2). To compare this with the $^{17}$O result, we note that, in the absence of librational averaging, $A_i(^{17}$O)/$A_i(^2$H) = 1.3 (Denisov & Halle, 1995), whence we can conclude that $N_i(^2$H) > $N_i(^{17}$O), as anticipated. Indeed, the quantitative analysis of the $^{17}$O data indicated that only three of the four internal water molecules of BPTI contribute to $^2$H, the fourth one, probably $W_{122}$ (Wlodawer et al., 1987), exchanging slowly on the $^2$H relaxation time-scale. If this water molecule exchanges rapidly on the (longer) $^2$H relaxation time-scale, we can estimate its generalized orientational order parameter: $A_{W_{122}}(^2$H) = 1.1(±0.1), corresponding to nearly complete orientational order (no librational averaging). Since it forms four hydrogen bonds to main-chain atoms (Wlodawer et al., 1987), $W_{122}$ is, indeed, expected to be highly ordered (Levitt, 1983). Since this internal water molecule contributes to the $^2$H relaxation but not to the $^{17}$O relaxation, the fast-exchange condition $r_{2H} > 1/R_i$ implies that its residence time is in the range 4 to 200 microseconds (see above).

The residence times for the internal water molecules of BPTI deduced here and in the preceding paper (Denisov & Halle, 1995) are much shorter than previous upper bound estimates (Tüchsen et al., 1987; Otting et al., 1991). It is interesting to note that our internal water residence times are much shorter than the residence times of the peptide ND deuterons that act as donors in hydrogen bonds with the internal water molecules. Using rate constants (corrected as in Table 2) for hydrogen exchange in Tyr10, Cys14, and Lys41 (Tüchsen et al., 1987), hydrogen-bonded to W112, W122, and W113, respectively, one finds peptide ND deuterons residence times as long as 0.13, 0.42, and 0.088 second, respectively, even at pH 11. These peptide deuterons experience anywhere from $10^0$ to $10^{14}$ (depending on pH) water exchanges before they are themselves exchanged.

### Concluding Discussion

Over the past 25 years it has usually been taken for granted that the water $^1$H and $^2$H relaxation rates are not significantly affected by rapidly exchanging protein hydrogen atoms in the neutral pH range 5 to 8. That labile protein hydrogen atoms contribute to the water $^1$H and $^2$H longitudinal relaxation outside this pH range has never been contested; this point has been verified experimentally for proteins (Koenig & Schillinger, 1969; Piculell & Halle, 1986) as well as for related polymer and colloidal systems (Halle & Piculell, 1982; Lankhorst & Leyte, 1984; Piculell, 1985, 1986). However, the case of the neutral pH range is not so clear-cut. The observation of frequency-dependent $^{17}$O relaxation in aqueous protein solutions (Halle et al., 1981) and the observation of a $^2$H relaxation dispersion for dimethyl sulfoxide in a mixed-solvent protein solution (Bryant & Jarvis, 1984) both demonstrate that water-protein hydrogen exchange is not the only contribution to the $^1$H and $^2$H relaxation dispersions seen in aqueous protein solutions. The similarity of the normalized $^1$H, $^2$H, and $^{17}$O relaxation rates in a lysozyme solution argues against a dominant contribution from protein hydrogen atoms in the neutral pH range (Koenig et al., 1975), as does the relatively weak pH dependence in the range 5 to 8 of $r_i(^1$H) in an apo-transferrin solution (Koenig & Schillinger, 1969). With regard to the latter result, however, it must be remembered that the quantitative interpretation of $^1$H relaxation data is complicated by cross-relaxation effects; it has even been argued that labile protein protons dominate the water $^1$H relaxation in protein solutions (Hills, 1992).

In contrast to $^1$H relaxation, the relaxation of the quadrupolar $^1$H and $^{17}$O nuclei is not significantly affected by magnetic dipolar couplings to protein protons, and is therefore much simpler to interpret. By measuring the $^2$H relaxation dispersion over a wide pH range, comparing it with the $^{17}$O dispersion from the same samples, and analyzing these relaxation data in terms of the known acid dissociation constants and hydrogen exchange rate constants of BPTI, we have, for the first time, obtained a quantitative picture of the effect of water-protein hydrogen exchange on the $^2$H relaxation dispersion.

In the only previous study that directly addressed the issue of water-protein deuteron exchange effects on longitudinal $^1$H relaxation, the pH dependence of the high-field $^1$H and $^{17}$O longitudinal and transverse relaxation rates from a lysozyme solution were compared (Piculell & Halle, 1986). This clearly demonstrated that the two nuclei do not monitor the same molecular species in the pH range 2 to 7. However, since the importance of the four internal water molecules of lysozyme (Blake et al., 1983) for the relaxation was not appreciated at that time, an
assessments of the labile protein deuteron contribution could not be made.

Since the $^{17}$O dispersion and, hence, the water contribution to the $^2$H dispersion is due to a few internal water molecules (Denisov & Halle, 1995), it is clear that even a small number of rapidly exchanging protein deuterons can make a contribution to the $^2$H dispersion ($\beta$ term) comparable to the water contribution. In contrast, the high-frequency relaxation enhancement ($\alpha$ term) is less sensitive to hydrogen exchange, since the water contribution to $\alpha$ is due to several hundred water molecules at the protein surface. At high pH, however, the large number of rapidly exchanging protein deuterons (approximately 50 in BPTI; see Figure 1(a)), with intrinsic high-frequency relaxation rates an order of magnitude larger than for surface water molecules (see Table 3), make a contribution to $\alpha$ comparable to that from surface water molecules (see Figures 7 and 8).

In the case of BPTI, the protein deuteron contribution to $\beta$ is negligible in the narrow pD range 5 to 6 (see Figure 1), since there are no Asp or Glu residues with a $p_D$ value higher than 4.2 (see Table 1). In many other proteins, however, local electrostatic interactions can increase carboxylic acid $pK_a$ values by several units. In addition, hydroxyl hydrogen exchange rate constants somewhat larger than the few directly determined ones for BPTI (Liepinsh et al., 1992) could occur in other proteins. On statistical grounds, these possibilities seem quite likely in larger proteins, e.g. serum albumin with 98 carboxyl groups (compared to five in BPTI) and 76 hydroxyl groups (eight in BPTI). Even for the small protein ubiquitin, our results indicate that labile protein deuterons contribute at least as much as water to the $^2$H dispersion at all pD values (see Figure 8). Finally, it should be remembered that the product $\tau R_i$ is strongly temperature-dependent, making the protein deuteron contribution more important at higher temperatures. (Water-protein hydrogen exchange is also more important for $^3$H relaxation, since $R_i(1^H)$ is an order of magnitude smaller than $R_i(2^H)$.) In conclusion, a careful assessment of hydrogen exchange is necessary in each case before reliable conclusions about protein hydration can be drawn from $^3$H relaxation data.

If studied systematically, the complexity of $^2$H relaxation can be turned into an advantage, since, in principle, it contains more information than $^{17}$O relaxation. As shown here (averaged) information about orientational order and internal motions of side-chains and surface peptide groups can be extracted from the $^2$H relaxation. Furthermore, due to its slower spin relaxation, the $^2$H nucleus has a wider dynamic range than $^{17}$O with regard to water exchange, allowing detection of internal water molecules with residence times in the range between $1/R_i(2^{17}$O) and $1/R_i(2^$H), typically $10^{-4}$ to $10^{-8}$ second. In the case of BPTI, the $^2$H and $^{17}$O relaxation data of the present and preceding studies indicate that the three internal water molecules W111 to W113 have residence times in the range $10^{-8}$ to $10^{-4}$ second, while the deeply buried water W122 has a longer residence time in the range $10^{-4}$ to $10^{-8}$ second.

In conclusion, we believe that the present work demonstrates that a combined $^2$H/$^{17}$O relaxation dispersion study can yield detailed dynamic information, currently not available by any other method, about buried water molecules and surface residues in globular proteins.

### Materials and Methods

#### Protein solutions

Recombinant bovine pancreatic trypsin inhibitor (M 6500 g mol$^{-1}$), a gift from Novo Nordisk A/S, was obtained as a lyophilized powder (Aprotinin, batch no. A46R02, 99.5% purity). Ubiquitin (M 8560 g mol$^{-1}$) from bovine red blood cells was obtained from Sigma as a lyophilized powder (catalogue no. U6253). The purity of the ubiquitin preparation was assessed by gel electrophoresis, indicating less than 1% contaminating protein. Both proteins were used as supplied, without further purification.

Protein solutions were made from heavy water (M 21.5 g mol$^{-1}$), enriched in $^{17}$O (Ventron, 21.9 atom% $^{17}$O, 61.9 atom% $^{18}$O, 9.95 atom% $^2$H). pH was measured with a Radiometer PHM63 digital pH-meter equipped with a 5 mm combination electrode. The direct reading pH$^*$ from a $^{3}$H$_2$O solution (with the pH meter calibrated with standard H$_2$O buffers) was converted to thermodynamically meaningful pD values according to pD = pH$^*$ + 0.41 (Covington et al., 1968). Desired pD values were obtained by adding small amounts of 5 M HCl or 5 M KOH to the protein solutions.

Protein concentrations were determined by complete amino acid analysis, which also confirmed the high purity of the protein preparations (see above) and demonstrated a negligible loss of terminal Gly, dipetide in ubiquitin. The protein concentrations obtained in this way were 10.4(±0.2)% (w/w) BPTI and 10.2(±0.2)% (w/w) ubiquitin. The small (<5%) variation of protein concentration due to pD adjustments was compensated for in the data analysis.

#### Relaxation dispersion measurements

Deuteron relaxation rates were measured at eight magnetic field strengths: (1) at 8.5 T on a Nicolet 360 spectrometer; (2) at 7.0 T on a Varian Unity 300 spectrometer; (3) at 2.35 T on a Bruker MSL 100 spectrometer; and (4) at 1.83, 1.505, 1.05, 0.7, and 0.45 T using an iron magnet (Drusch EAR-35N) equipped with field-variable lock and flux stabilizer and operated from the MSL 100 spectrometer. The sample temperature was adjusted to 27.00(±0.05)°C using a copper-constantan thermocouple, and was maintained to within ±0.05 deg. C (±0.1 deg. C on the Varian spectrometer) by a thermostated air flow. NMR tubes with an outer diameter of 10 mm were used, with sample volumes ranging from 0.7 to 1.7 ml.

Longitudinal relaxation rates were measured by the inversion recovery pulse sequence (180°-τ-90°). The 180° pulse angle was calibrated to within 1° before each measurement, and the 90° pulse length was set to half that of the 180° pulse. Phase cycling was used to eliminate residual pulse length and phase errors. At least 16 transients (one full phase cycle) were accumulated for each τ value. Each relaxation experiment comprised 20 τ values (in random order) in the interval (0.05 to 5.3)°T. The
longitudinal relaxation rate $R_L = 1/T_1$ was determined from an exponential fit to the 20 data points using standard software. The accuracy of the determined relaxation rates was estimated to be better than 1%. The longitudinal relaxation rate for bulk water of the same isotopic composition as in the protein solutions was measured 12 times within several months and at different fields, with the result: $R_L = 2.26(\pm 0.22) \text{ s}^{-1}$.

Non-linear fits of theoretical expressions (see Results and Discussion) to the $R_f$ dispersion data were made with the Levenberg-Marquardt algorithm.

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