Direct Observation of Calcium-Coordinated Water in Calbindin D_{9k} by Nuclear Magnetic Relaxation Dispersion

Vladimir P. Denisov* and Bertil Halle

Contribution from the Condensed Matter Magnetic Resonance Group, Lund University, Chemical Center, P.O. Box 124, S-22100 Lund, Sweden

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Abstract: The frequency dispersions of the water ^{17}O and ^{2}H nuclear magnetic relaxation rates have been measured in solutions of the calcium-binding protein calbindin D_{9k} in the apo and calcium-loaded states. The relaxation data show that the residence times of the two water molecules that ligate calcium ions in the crystal structure are in the range 5 ns to 7 μs, much longer than for calcium-coordinated water in bulk solution. In addition to a twist libration of substantial amplitude, the calcium-coordinated water molecules in calbindin undergo a fast (<1 ns) flip motion, resulting in a drastic reduction of the ^{2}H dispersion amplitude. The residence time as well as the internal motions of these water molecules are largely governed by strong hydrogen bonds to side chains that may be essential for the cooperativity of calcium binding. In addition to the calcium-coordinated waters, calbindin contains (at least) one long-lived (>5 ns) water molecule, which we tentatively identify as a structure-stabilizing water molecule buried in a surface pocket near the linker loop. Even at pD 6.7, the ^{2}H relaxation dispersion is dominated by rapidly exchanging carboxyl deuterons in highly ordered side chains. The present study provides the first direct observation by means of NMR of water molecules coordinated to a diamagnetic metal ion in a protein solution.

Introduction

Many proteins rely on intrinsic divalent metal ions to carry out catalytic, regulatory, or other physiological functions.[1] Accordingly, the study of the geometry, energetics, and dynamics of metal ion binding sites in proteins is an active field,[2−5] with particular emphasis on calcium-binding proteins.[6−9] The most common diamagnetic metal ions in proteins, Ca^{2+}, Zn^{2+}, and Mg^{2+}, often ligate one or several water molecules. In some proteins such metal-coordinated waters are directly involved in the catalytic mechanism; in other proteins they are used to fine-tune the affinity and kinetics of biofunctional metal-ion binding.

While structural water molecules, buried within small globular proteins, have recently been identified by high-resolution multidimensional NMR[10] spectroscopy[11−19] and by water ^{17}O and ^{2}H nuclear magnetic relaxation dispersion[20−23] (NMRD), water molecules bound to diamagnetic metal ions in protein solutions have not, to our knowledge, previously been observed or characterized by NMR or any other technique. In fact, due to the scarcity of NOE constraints, the geometry of metal-binding sites of proteins in solution is often incompletely characterized also with regard to non-water ligands. In some cases, complementary information can be obtained by metal-ion NMR[24,25] or by other specialized NMR techniques.[26] In addition, water relaxation studies can provide detailed information about metal-coordinated water in paramagnetic proteins.[27,28] In the case of diamagnetic proteins, however, the only previous water relaxation study failed to observe metal-coordinated water.[29]

Recent water ^{17}O and ^{2}H NMRD studies[20−23] have established that even a single structural water molecule can significantly enhance the low-frequency relaxation of the bulk water signal, provided that (*i*) it has a residence time in the range 10^{-8}−10^{-6} s (for ^{17}O) or 10^{-8}−10^{-4} s (for ^{2}H) and (*ii*) it has a

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relatively high orientational order parameter. These requirements appear to be satisfied by most waters classified as "internal" (i.e., not hydrogen bonded to external water) in high-resolution crystal structures,[30-34] as well as by a few waters buried in narrow pockets or crevices at the protein surface. The present study was motivated by the expectation that the residence times and order parameters of metal-bound waters are in the same range as for the previously investigated, buried structural waters. The resulting water ^{17}O and ^{2}H relaxation dispersions would then provide new information about the residence times and internal motions of metal-bound water molecules that may be biofunctionally relevant. For this purpose, we chose to study the calcium-binding protein calbindin D_{9k}.

Like the other members of the calmodulin superfamily of regulatory, signaling, or buffering calcium-binding proteins, calbindin D_{9k} binds calcium ions with high affinity and selectivity to sites formed by a highly conserved helix–loop–helix structural motif, termed the EF hand.[6-9,25,35] The EF hand is one of the most prevalent structural binding motifs found in nature, and a detailed characterization of its coordination geometry, energetics, and dynamics is clearly a prerequisite for understanding the delicate tuning of the intricate network of calcium fluxes and signals in biological cells. Calbindin D_{9k}, with 75 residues, contains a pair of EF hands that bind two calcium ions with positive cooperativity.[36-38] The N-terminal site I is two residues longer than the archetypal C-terminal site II. In the crystal structure, each calcium ion has an approximately pentagonal bipyramidal coordination of 7 oxygen ligands, one of which belongs to a water molecule.[39,40] While the calcium coordination of calbindin D_{9k} in solution has not been characterized in detail, recent NMR studies[41-47] demonstrate that the average backbone conformation is virtually the same in solution as in the crystal and that calcium binding induces only minor conformational changes.

By recording the water ^{17}O and ^{2}H NMRD profiles from calbindin solutions as a function of calcium loading, we show here that the two water molecules that ligate calcium ions make a dominant contribution to the ^{17}O relaxation dispersion. The residence times of these two water molecules are in the range 5 ns to 7 μs, much longer than for the waters in the primary coordination shell of a calcium ion in bulk solution. This difference is presumably due to strong hydrogen bonds, evident in the crystal structure, between the calcium-coordinated waters in calbindin and several side chains that may be crucial for the observed positive cooperativity of calcium binding.[36-38] The combined ^{17}O and ^{2}H NMRD data show that, although the calcium-coordinated water molecules are prevented by the hydrogen bonds from rotating freely around the 2-fold axis, they undergo a fast (< 1 ns) flip motion around this axis. The NMRD data show that at least one additional water molecule has a residence time exceeding 5 ns. We tentatively assign this contribution to a partly buried water molecule near the Leu39–Ser44 linker loop.[39,40] Whereas the ^{17}O dispersion reports exclusively on water molecules, the ^{2}H dispersion is found to be dominated by rapidly exchanging carboxyl deuterons even at neutral pD.

Materials and Methods

Materials. Calbindin D_{9k} was expressed in *E. coli* from a synthetic gene and purified as described elsewhere.[48] The protein represents the wild-type minor A form of bovine calbindin D_{9k} with an additional methionine residue (Met0) at the N-terminus; it thus contains 76 amino acid residues (MW 8615 g mol^{-1} at pD 6.7). The lyophilized protein was dissolved in heavy water (MW 21.5 g mol^{-1}), enriched in ^{17}O (Ventron; 21.9 atom % ^{17}O, 61.9 atom % ^{18}O, 99.95 atom % ^{2}H). The calcium loading of the protein was varied by addition of 1 M $CaCl_2$ directly into the NMR tube. For the fully loaded form, the total calcium concentration was ca. 3 mol of Ca^{2+}/mol of protein. The residual calcium content of the apo protein and that of the partially loaded protein were determined spectrophotometrically from Ca^{2+} titrations with the chelator Quin 2.[37]

Solution pH was measured with a Radiometer PHM63 digital pH-meter equipped with a 5-mm combination electrode. The direct reading pH* from a D_2O solution (with the pH meter calibrated with standard H_2O buffers) was converted to pD values according to pD = pH* + 0.41.[49] pD was adjusted by adding minute amounts of 1 M HCl or 1 M NaOH to the protein solution.

The protein concentration was determined by complete amino acid analysis, which confirmed the high purity of the protein preparation and indicated some loss of terminal Met0 residue.

Relaxation Dispersion Measurements. Oxygen-17 and deuteron relaxation rates were measured at eight magnetic field strengths: at 7.0 T on a Varian Unity 300 spectrometer, at 4.7 T on a Bruker DMX 200 spectrometer, at 2.35 T on a Bruker MSL 100 spectrometer, and 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 maintained at 27.0 ± 0.1 °C by a thermostated air flow.

The longitudinal ^{17}O and ^{2}H relaxation rates, R_1, were measured as described previously.[21,22] For some samples, the transverse relaxation rate, R_2, was also measured. Due to additional contributions to R_2, particularly significant for the ^{2}H rate, only R_1 data were included in the analysis.[21] Nonlinear fits of the parameters in eq 1 to the R_1 dispersion data were made with the Levenberg–Marquardt algorithm.

Results and Discussion

Calcium-Dependent Water Relaxation Dispersion. The longitudinal relaxation rate of water ^{17}O and ^{2}H nuclei in aqueous protein solutions exhibits a dispersion in the megahertz range, due to water molecules associated with the protein for periods long compared to the rotational correlation time, τ_R, of the protein.[50,51] In addition, the ^{2}H rate may be influenced by

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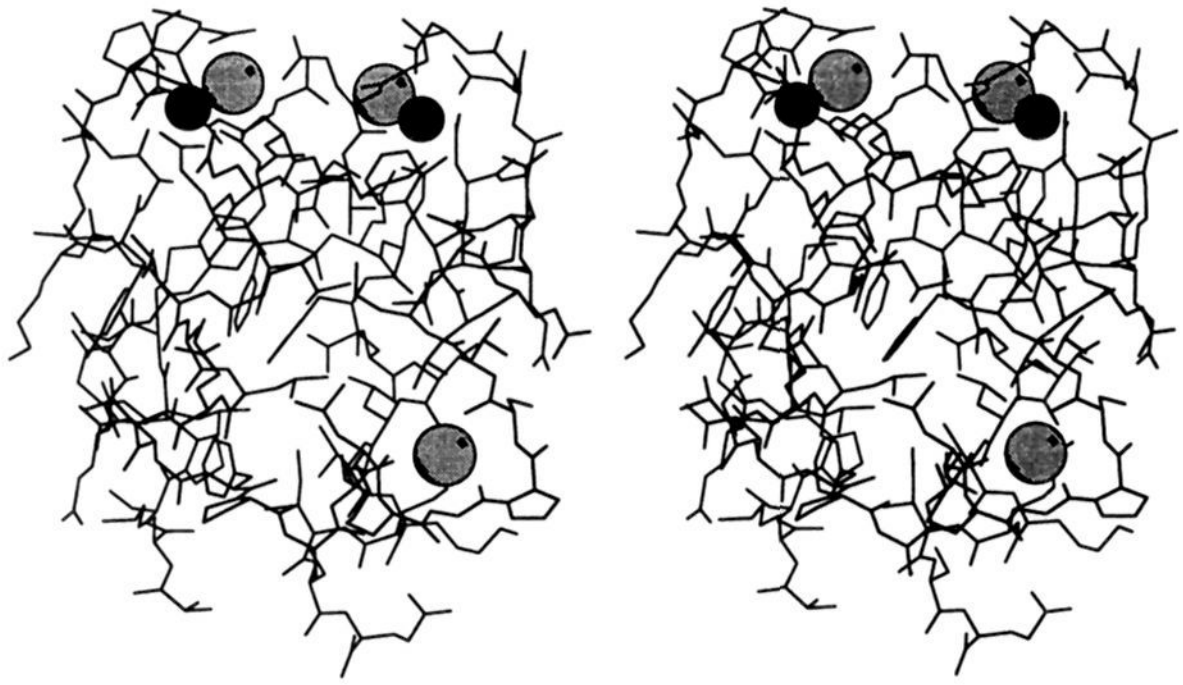

Figure 1. Stereo view of the calbindin D_{9k} crystal structure[40] (Protein Data Bank, file 4ICB). Regions with two conformations are represented in the A conformation (trans isomer of the Gly42−Pro43 peptide bond). The location of the calcium ions (dark spheres of radius 1.0 Å) and of the oxygen atoms of the water molecules W78, W79, and W86 (grey spheres of radius 1.4 Å) are shown. The drawing was made with the program Molscript.[88]

deuteron exchange between water and labile protein hydrogens.[22] We have recently demonstrated that only internal water molecules are sufficiently long lived to contribute substantially to the relaxation dispersion in solutions of bovine pancreatic trypsin inhibitor (BPTI).[20,21] For the protein ubiquitin, without internal waters, we found only a very small dispersion step, attributed to a single moderately ordered, water molecule, residing in a surface pocket. Calbindin D_{9k} and ubiquitin have virtually the same molecular mass, both have a nearly spherical global shape, and both have a densely packed core, devoid of deeply buried water molecules.[34,52]

According to the crystal structure,[39,40] calbindin D_{9k} contains one water molecule with four main-chain hydrogen bonds in a surface pocket, while each of the two bound calcium ions ligates one water molecule (see Figure 1). On the basis of our previous results,[20−23] these three water molecules are the most likely candidates for an ^{17}O relaxation dispersion. The ^{2}H relaxation rate is expected to contain also a contribution from labile protein hydrogens even at neutral pH, since the apo protein has several Glu residues with exceptionally high pK_a values (Kesvatera et al., to be published).

Figures 2 and 3 show (some of) the ^{17}O and ^{2}H relaxation dispersion profiles from D_2O solutions of calbindin D_{9k} at different levels of calcium loading. The data are accurately represented by the classical theoretical expression[53,54]

$$R_1(\omega_0) = R_{bulk} + \alpha + \beta\tau_R[0.2/(1 + \omega_0^2\tau_R^2) + 0.8/(1 + 4\,\omega_0^2\tau_R^2)] \quad (1)$$

where $\omega_0 = 2\pi\nu_0$ is the Larmor (angular) frequency and τ_R (usually equal to the rotational correlation time of the protein) is the effective correlation time for the long-lived water molecules, giving rise to the dispersion amplitude β. Furthermore, R_{bulk} is the bulk water relaxation rate and α is the frequency-independent relaxation contribution from the short-lived water molecules at the protein surface and from fast internal motion of the long-lived waters. The curves in Figures 2 and 3 resulted from nonlinear least-squares fits of the three parameters α, β, and τ_R in eq 1 to the eight data points of each dispersion curve.

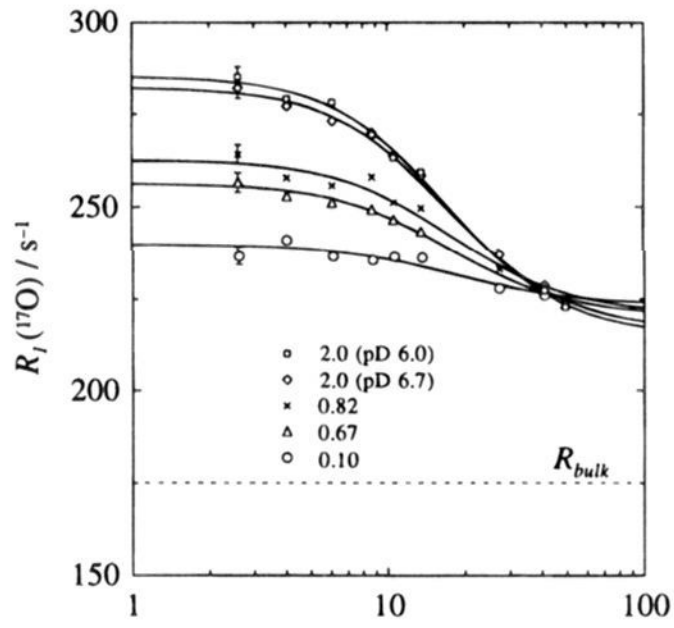

Figure 2. Dispersion of the water ^{17}O longitudinal relaxation rate in D_2O solutions (27 °C) of calbindin D_{9k} at the indicated values of the average calcium loading, N_{Ca} (mol of Ca^{2+}/mol of calbindin). Protein concentrations and pD values are given in Table 1. [The data from the sample with $N_{Ca} = 0.67$ were scaled from 6.9 to 6.0 wt %, assuming that the excess relaxation rate, $R_1 - R_{bulk}$, is proportional to $w/(1 - w)$; cf. eq 2.] The estimated experimental error of ±1% is indicated at the lowest field. The dashed line refers to the bulk solvent ^{17}O relaxation rate.

The rotational correlation times, τ_R, obtained from the dispersion fits cluster around 5.0 ns with an uncertainty of ca. 0.5 ns. We find a slight (barely significant) increase of τ_R on calcium binding (4.8, 5.0, and 5.3 ns for 0.1, 0.8, and 2 bound Ca^{2+} ions, respectively). These results are in excellent agreement with previous determinations, when the hydrogen and oxygen isotope effects on the solvent viscosity are taken into account. ^{15}N relaxation data at pH 6.0 and 27 °C thus yielded $\tau_R = 4.10 \pm 0.01$ ns (5.19 ns) for the apo state[42] and $\tau_R = 4.25 \pm 0.04$ ns (5.38 ns) for the fully loaded state,[43] the values within parentheses being scaled to our solvent viscosity (1.09 cP). Since these values were obtained at protein concentrations (4 and 5 mM) roughly half of that used here, we conclude that protein−protein interactions do not significantly affect τ_R at protein

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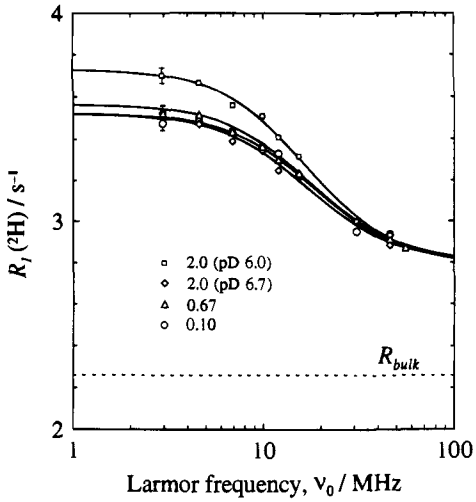

Figure 3. As in Figure 2, but for ^{2}H.

Table 1. Parameters Derived from ^{2}H and ^{17}O NMRD Data from Calbindin D_{9k} Solutions at 27 °C

			α (s^{-1})$^{c-e}$		β (10^9 s^{-2})$^{c-e}$	
N_{Ca}[a]	pD	C_P (mM)[b]	^{2}H	^{17}O	^{2}H	^{17}O
0.10	6.67	8.4	0.55	48	0.138	3.0
0.67	6.71	9.5	0.54	46	0.143	6.8
0.82		8.3	0.56	47	0.149	7.8
2.00		9.3	0.51	41	0.175	13.2
2.00	6.03	8.2	0.54	43	0.177	12.3
2.00	6.68	8.1	0.54	45	0.133	12.1

[a] Average calcium loading in NMR sample, mole of Ca^{2+}/mole of calbindin. [b] Protein concentration in NMR sample. [c] From 2-parameter fits as described in the text. [d] Corrected to 6.0 wt % calbindin (8.3 mM), assuming that α and β are proportional to $w/(1-w)$; cf. eq 2. [e] Errors from fits are as follows: ±0.025 for α(^{2}H), ±2 for α(^{17}O), ±0.007 for β(^{2}H), and ±0.5 for β(^{17}O).

concentrations up to ca. 8 mM. The quoted rotational correlation times are also consistent with the results, $\tau_R = 3.7 \pm 0.5$ ns (4.5 ns) for the apo state and $\tau_R = 4.2 \pm 0.7$ ns (5.1 ns) for the fully loaded state, deduced from fluorescence spectroscopy (at pH 8).[55]

Since our primary interest here is in α and β, rather than in τ_R, we also fitted these two parameters while keeping τ_R fixed at the more precise values derived from ^{15}N relaxation.[42,43] The resulting dispersion curves are indistinguishable from those shown in Figures 2 and 3 and the new α and β values are within the error limits of the original values. Due to a significant covariance of α and β with τ_R in the 3-parameter fit, however, the errors from the 2-parameter fit are smaller. The α and β values derived in this way are collected in Table 1 and plotted versus calcium loading in Figures 4 and 5.

The ^{17}O relaxation dispersion amplitude for apo calbindin (the lowest curve in Figure 2) is very small, as previously observed with ubiquitin.[21] This similarity is indeed expected from the absence of deeply buried waters in both crystal structures. When scaled to the same protein concentration, the dispersion amplitude, β, of apo calbindin is nearly twice that of ubiquitin, consistent with at least one long-lived and well-ordered surface water. The strong dependence of the ^{17}O dispersion amplitude on calcium loading (cf. Figures 2 and 5a) demonstrates that calbindin gains additional long-lived water molecules on binding of the two Ca^{2+} ions.

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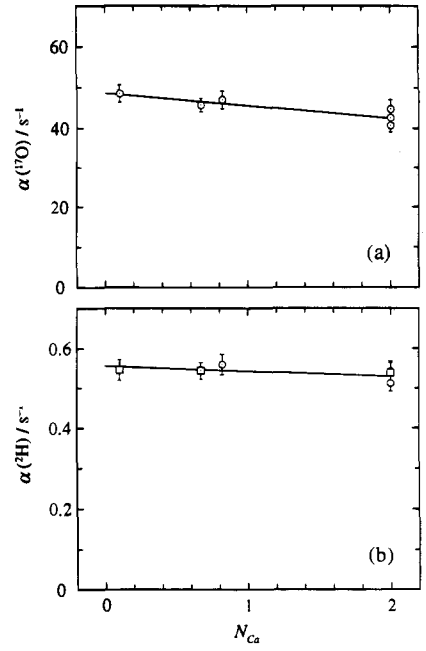

Figure 4. Variation of the frequency-independent relaxation parameter α, for (*a*) ^{17}O and (*b*) ^{2}H, with the average calcium loading, N_{Ca}, of calbindin D_{9k}. For the ^{2}H data, circles refer to constant ionization and squares to constant pD.

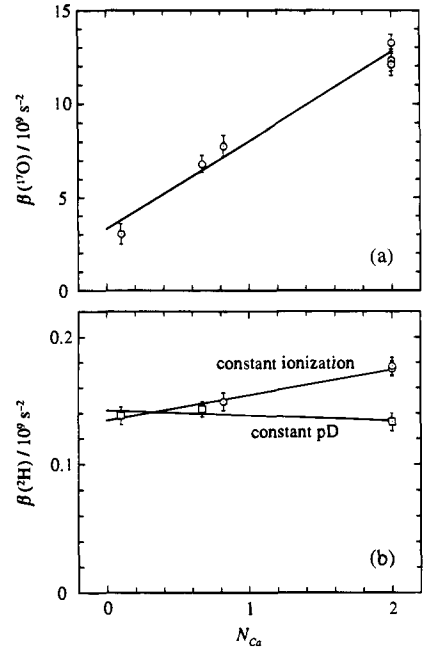

Figure 5. Variation of the dispersion amplitude β, for (*a*) ^{17}O and (*b*) ^{2}H, with the average calcium loading, N_{Ca}, of calbindin D_{9k}. For the ^{2}H data, circles refer to constant ionization and squares to constant pD.

In contrast to the ^{17}O data, the ^{2}H relaxation exhibits a strong dispersion even for the apo protein (cf. Figure 3), indicating that the contribution from labile protein hydrogens to the solvent relaxation rate is dominant even at neutral pD. A significant labile hydrogen contribution at neutral pD was also found for ubiquitin.[22] On the other hand, calcium binding has a much smaller effect on the ^{2}H dispersion than on the ^{17}O dispersion (cf. Figure 5). This observation implies that the calcium-coordinated water molecules are not irrotationally bound with respect to the protein on the time scale, τ_R, of its tumbling. To

quantitatively interpret the ^2H data, we must take into account the variable degree of ionization of the Asp and Glu residues of calbindin, responsible for the significant difference in the dispersion amplitude β between pD 6.0 and 6.7 (cf. Figures 3 and 5b).

Calcium-Coordinated Water Molecules. The variation of the dispersion amplitudes $\beta(^{17}O)$ and $\beta(^2H)$ with the average number, N_{Ca}, of bound Ca^{2+} ions per calbindin molecule (cf. Figure 5) can be quantitatively interpreted in terms of the expressions[21,22,54]

$$\beta(^{17}O) = (12\pi^2/125)(M_W/M_P)[w/(1-w)]N[A(^{17}O)\chi(^{17}O)]^2 \quad (2a)$$

$$\beta(^2H) = (3\pi^2/2)(M_W/M_P)[w/(1-w)]N[A(^2H)\chi(^2H)]^2 \quad (2b)$$

where M_W and M_P are the molar mass of water and protein, and w is the protein mass fraction. Furthermore, N is the number of long-lived water molecules, with (nucleus-specific) generalized order parameter A and quadrupole coupling constant χ, that contribute significantly to the relaxation dispersion.

Since calcium binding has been shown to induce only small changes in the solution structure of calbindin,[41-47] we ascribe the variation of β with N_{Ca} to calcium-coordinated water molecules, i.e., we write $N = N_{Ca}$, assuming for the moment that each of the two calcium-coordinated water molecules contribute to β (cf. below). Due to the positive cooperativity of calcium binding to calbindin, the fraction of protein molecules with a single bound Ca^{2+} ion is small for any N_{Ca}.[36-38] Consequently, we expect β to increase linearly with N_{Ca} according to eq 2. From the slope of the fitted lines in Figure 5 (constant ionization for ^2H; cf. below), we thus obtain $A(^{17}O)\chi(^{17}O) = 5.6 \pm 0.2$ MHz and $A(^2H)\chi(^2H) = 92 \pm 9$ kHz. These figures represent averages over the two calcium-coordinated water molecules.

To proceed, we need the quadrupole coupling constants, $\chi(^2H)$ and $\chi(^{17}O)$, for the calcium-coordinated water molecules in calbindin. The values, $\chi(^2H) = 213$ kHz[56] and $\chi(^{17}O) = 6.5$ MHz,[57,58] measured in ice Ih, are appropriate for internal water molecules with four hydrogen bonds.[21,22] A realistic estimate of $\chi(^2H)$ for the calcium-coordinated water molecules in calbindin can be obtained from experimental data on gypsum, $CaSO_4 \cdot 2H_2O$, where the water molecules, with a calcium-oxygen distance of 2.4 Å and two strong hydrogen bonds to sulfate oxygens,[59] have a coordination geometry nearly identical with that in calbindin. (This point is elaborated below.) ^2H NMR studies of gypsum yield an effective ^2H quadrupole coupling constant of 117.3 ± 0.3 kHz[60,61] at room temperature, where the water molecules flip rapidly (compared to the quadrupole frequency) around the 2-fold axis.[62] The unaveraged quadrupole coupling constant $\chi(^2H)$ is obtained by dividing the reported value by $(1+\eta)/2$, with η the asymmetry parameter of the (unaveraged) electric field gradient tensor.[63] In ice Ih, $\eta = 0.11$.[56] Taking $\eta = 0.1 \pm 0.1$, we thus obtain $\chi(^2H) = 213 \pm 19$ kHz for gypsum, the same value as in ice Ih. For the numerous crystal hydrates and ice polymorphs where both have been measured, it is found that $\chi(^2H)$ and $\chi(^{17}O)$ exhibit a strong (linear) correlation.[64] Consequently, also $\chi(^{17}O)$ should be essentially the same in gypsum and in ice Ih. These experimentally derived estimates are in accord with quantum-chemical calculations,[51,54,65] showing that the reduction of χ arising from polarization of the water molecule by hydrogen bonds is roughly the same as that due to a coordinating ion. For the following calculations we shall thus use the ice Ih values for $\chi(^2H)$ and $\chi(^{17}O)$, allowing for 10% uncertainty in both.

Assuming that one water molecule ligates each Ca^{2+} ion (as in the crystal) and that each of these water molecules contribute to β, we obtain the generalized order parameters $A(^2H) = 0.43 \pm 0.06$ and $A(^{17}O) = 0.86 \pm 0.09$. An upper bound for the generalized order parameter is set by the rigid-lattice value $A = (1+\eta^2/3)^{1/2}$, which is 1.00 for ^2H and 1.13 for ^{17}O.[56-58] A lower value of A implies internal motion of the water molecule with respect to the protein on a time scale short compared to $\tau_R = 5$ ns. If only one calcium-coordinated water molecule contributes to β, we obtain instead $A(^2H) = 0.61 \pm 0.08$ and $A(^{17}O) = 1.22 \pm 0.13$. Thus $A(^{17}O)$ becomes (slightly) larger than the rigid-lattice value, indicating that both calcium-coordinated water molecules contribute to β. This interpretation is strengthened by the ensuing analysis of internal motions.

Since the electric field gradient tensors at the ^2H and ^{17}O nuclei in the water molecule have different principal axis orientations and different asymmetries, $A(^2H)$ and $A(^{17}O)$ are in general affected differently by a given mode of internal motion.[54] In the Appendix, we derive explicit expressions for $A(^2H)$ and $A(^{17}O)$ for librational motions around each of three orthogonal axes: plane libration (restricted rotation around the normal to the molecular plane), wag libration (restricted tilting of the molecular plane), and twist libration (restricted rotation around the C_2 axis of the water molecule). Using these expressions, we have calculated the variation of $A(^2H)$ and $A(^{17}O)$ with the libration amplitude ϕ_0, assuming that the libration angle ϕ is uniformly distributed in the interval $-\phi_0 < \phi < \phi_0$. As seen from Figure 6, none of these libration modes (and, presumably, no combination of them) can simultaneously account for the experimental values of $A(^2H)$ and $A(^{17}O)$.

Several NMR studies have shown that water molecules in crystal hydrates undergo 180° flips around the C_2 axis at rates that depend strongly on the environment.[66] Whereas the C_2 flip does not affect $A(^{17}O)$, it reduces $A(^2H)$ from the rigid-lattice value of 1.00 to 0.59 (cf. the Appendix). As seen from Figure 6, the experimental values of $A(^2H)$ and $A(^{17}O)$ can be accounted for only if the calcium-coordinated water molecules perform C_2 flips. To be effective, this motion must be fast compared to $\tau_R = 5$ ns. A quantitative agreement with the A values derived on the assumption that both calcium-coordinated water molecules contribute to β requires, in addition to a C_2 flip, also a twist libration of 37° amplitude (cf. the dotted lines in the lower panel of Figure 6). As further discussed below, structural and energetic considerations argue for a librational motion of predominantly twist character and of relatively large amplitude. On the other hand, a free rotation ($\phi_0 = 180°$) around the C_2 axis is ruled out, since this would severely reduce the dispersion amplitude for both ^2H and ^{17}O (cf. Figure 6). As already noted, the possibility that only one of the two calcium-coordinated water molecules contributes to β is unlikely in view of the large $A(^{17}O)$. When we allow for a plausible twist libration, this possibility clearly becomes even less likely (cf. Figure 6).

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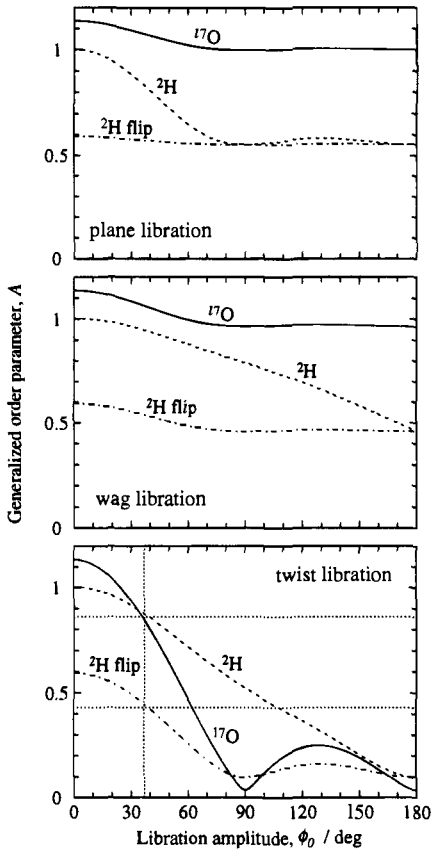

Figure 6. Variation of the generalized order parameters $A(^2H)$ and $A(^{17}O)$ with the libration amplitude for the three libration modes defined in the text. The effect of fast C_2 flips on $A(^2H)$ is also shown; $A(^{17}O)$ is unaffected. For the calculations, we have used $2\alpha = 104.5°$, $\eta(^2H) = 0.11$, and $\eta(^{17}O) = 0.93$. The order parameter curves are essentially unaffected by physically reasonable variations in these parameters. The bottom panel shows (dotted lines) the experimentally derived values of $A(^2H)$ and $A(^{17}O)$ for the calcium-coordinated water molecules in calbindin D_{9k}.

The finding that the calcium-coordinated water molecules in calbindin contribute to the ^{17}O relaxation dispersion implies that their mean residence times are longer than the rotational correlation time, $\tau_R = 5$ ns, of the protein, but shorter than their intrinsic relaxation time, $T_1(^{17}O)$. The latter quantity is obtained from the slope of the line in Figure 5a according to $1/T_1(^{17}O) = (M_P/M_W)[(1 - w)/w][d\beta(^{17}O)/dN_{Ca}]\tau_R$. The residence time must therefore be in the range

$$5 \text{ ns} < \tau_{res} < 7\ \mu s \qquad (3)$$

Long-Lived Water Molecules in the Apo Protein. The observation of an ^{17}O dispersion, albeit relatively weak, at $N_{Ca} = 0.1$ implies that apo calbindin contains at least one long-lived ($\tau_{res} > 5$ ns) water molecule. Our experience with other proteins suggests that any such water molecule should be tucked away in a narrow surface pocket, where it should engage in three or four hydrogen bonds to protein atoms. Setting $N = 1$ in eq 2a, inserting the β value given by the intercept of the line in Figure 5a, and taking $\chi(^{17}O) = 6.5$ MHz ($\pm 10\%$), (cf. above), we obtain $A(^{17}O) = 0.72 \pm 0.08$. The residence time of this water molecule must then be in the range 5 ns $< \tau_R <$ 10 μs. If there are several long-lived water molecules, their average order parameter is correspondingly smaller.

It is conceivable that $\beta(^{17}O)$ for apo calbindin is due to water molecules occupying the calcium-free binding sites. However, as these sites are delicately tuned for high Ca^{2+} affinity and specificity,[6-9] water molecules may well be excluded on steric and electrostatic grounds. In the only available high-resolution crystal structure of a calcium-free site in an EF-hand protein (troponin C), water molecules appear to be present.[67,68] However, these water-occupied sites are more flexible and have much lower Ca^{2+} affinity than the calcium sites of calbindin.[6-9]

While calbindin contains no deeply buried water molecules, both crystal structures show that one water molecule is buried in a pocket near the linker loop that connects the two EF hands.[39,40] This water molecule, denoted W86[40] and included in Figure 1, donates two strong hydrogen bonds (2.7 and 2.8 Å) to the carbonyls of Leu39 and Ser44 and accepts two weaker hydrogen bonds (3.1 and 3.4 Å) from the amide nitrogens of Gly42 and Ser44. Another candidate is water W81,[40] located in a pocket of loop II, where it donates two strong hydrogen bonds (2.6 and 2.7 Å) to Leu53 and Glu65 and accepts hydrogen bonds (both 3.0 Å) from N_ϵ of Lys55 and from an external water molecule. This water molecule is absent in the other crystal structure,[39] presumably due to the flexibility of the side chain of Lys55. Since W81 appears to be less shielded than W86 from the external solvent, we regard W86 as the most likely candidate.

Except for the C-terminus, the linker loop region, where W86 is located, is the most flexible part of calbindin, as judged from the temperature factors in the crystal structure[39,40] and the peptide NH order parameters in the solution structure.[42,43] However, the water molecule W86 does not appear to be disordered with respect to the protein atoms to which it hydrogen bonds, since it has essentially the same temperature factor (ca. 20 Å^2) as two of its hydrogen-bonded partners (Leu39 and Gly42). Furthermore, $A(^{17}O) = 0.7$ is not inconsistent with $0.7 < A_{NH} < 0.9$, as reported for Leu39, Gly42, and Ser44.[42,43] A long residence time is consistent with a structure-stabilizing role for W86, in accord with the general picture of structural waters being predominantly located in loop and turn regions, where they extend the regular secondary structure.[30-34] Although the amide nitrogen of Gly42, to which W86 is hydrogen bonded, changes position during the cis-trans isomerization of the Gly42–Pro43 peptide bond,[40,69] the rate of this process, ca. 0.2 s^{-1} at 25 °C,[70] is much too low to affect the residence time of W86.

Hydrogen Exchange and Side-Chain Order Parameters. In general, the ^2H excess relaxation is due not only to protein hydration but also to labile hydrogens exchanging rapidly between protein and bulk water.[22] In the pD range 6.0–6.7 investigated here, only the carboxyl hydrogens (Asp, Glu, and C-terminus) exchange sufficiently rapidly to contribute to the ^2H relaxation. Since all the 17 Asp and Glu residues of calbindin reside at the surface, they should have hydrogen residence times in the microsecond range,[71] two or three orders of magnitude shorter than the intrinsic ^2H relaxation time.[22]

Although most carboxyl groups are ionized in the neutral pH range, the accumulation of negative charge near the Ca^{2+} binding sites should produce some exceptionally high pK_a values in calbindin. Indeed, a recent determination of all the individual pK_a values in apo calbindin in H_2O (Kesvatera et al., to be

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Table 2. Ionization State of Carboxyl Groups in Calbindin D_{9k} in D_2O in the Apo and Fully Loaded States[a]

				N_{COOD} (in D_2O)		
class	no.	$\langle pK_a^H\rangle_{apo}$	$\langle pK_a^H\rangle_{2Ca}$	apo pD 6.67	loaded pD 6.03	loaded pD 6.67
ligands	5	5.70	<4	1.44	0	0
nearby	5	5.54	4.9	1.03	0.93	0.25
distant	8	4.98	4.98	0.77	2.31	0.77
total	18	5.34		3.24	3.24	1.02

[a] Based on individual pK_a values (Kesvatera et al., to be published) and Ca^{2+}-induced pK_a shifts as described in the text.

published) yields an average pK_a^H of 5.34 for the 18 carboxyl groups, three of them having $pK_a^H > 6$. For convenience, we divide these carboxyl groups into three classes: (*i*) 4 Asp and Glu residues whose carboxyl groups ligate Ca^{2+} (Asp54, Asp58, Glu27, and Glu65) plus Glu60, which, in the crystal structure, is strongly hydrogen bonded to the calcium-coordinated water molecule in site I, (*ii*) 5 additional Asp and Glu residues near the sites (Asp19, Glu11, Glu17, Glu51, and Glu64), and (*iii*) the 8 remaining carboxyl groups (including the C-terminus) that are further removed from the binding sites. Using the individual pK_a^H values (Kesvatera et al., to be published) and correcting for the solvent isotope effect according to[72] $pK_a^D = pK_a^H + 0.50$, we have calculated the ionization state of these three classes of carboxyl groups under the experimental conditions of the present study (see Table 2).

When apo calbindin is saturated by addition of $CaCl_2$, we find that the solution pD drops from 6.67 to 6.03. At a protein concentration of ca. 8 mM, this corresponds to a net dissociation of $<10^{-4}$ deuterons per calbindin molecule. Although the total number of labile deuterons in calbindin is thus independent of calcium loading, Ca^{2+}-induced pK_a shifts lead to a redistribution of labile deuterons among the carboxyl groups of calbindin. Assuming that the carboxyl groups in the ligand class are fully ionized in the calcium-loaded state and that the pK_a values of the distant class are unaffected by Ca^{2+} binding, we can reproduce the observed pD shift if the pK_a values of the nearby class are reduced by 0.65 unit on calcium binding. As seen from Table 2, the effect of Ca^{2+} binding is then to transfer 1.44 deuterons from the binding sites to other, mainly distant, carboxyl groups. When the calcium-loaded protein is titrated back to the original pD 6.67, 2.2 deuterons are removed, mainly from distant residues.

The ^2H dispersion amplitude can be decomposed as[22]

$$\beta(^2H) = \beta_W(^2H) + \beta_{COOD}(^2H) \quad (4)$$

The water contribution, $\beta_W(^2H)$, is given by eq 2b with $N = N_W$, the number of long-lived water molecules. The contribution, $\beta_{COOD}(^2H)$, from rapidly exchanging carboxyl deuterons is also given by eq 2b, but with $N = N_{COOD}/2$.

We consider first the difference in $\beta(^2H)$ between pD 6.0 and 6.7 for the calcium-loaded protein (cf. Figure 5b). The water contribution $\beta_W(^2H)$ cancels out in this difference, since, like $\beta(^{17}O)$, it should be independent of pD (cf. Figure 5a or Table 1). Subtracting the last two columns in Table 2, we obtain $\Delta N_{COOD} = 2.22$. With this value and $\beta(^2H)$ data from Table 1 inserted into eq 2b, we obtain $\langle A_{COOD}\chi_{COOD}\rangle = 130 \pm 15$ kHz. This represents an average over 0.68 nearby and 1.54 distant COOD groups.

For the apo protein, we can calculate $\beta_{COOD}(^2H)$ by subtracting from the measured $\beta(^2H)$ a value for $\beta_W(^2H)$ obtained by scaling $\beta(^{17}O)$ according to eq 2. With $\chi(^{17}O)/\chi(^2H) = 30.5$ (cf. above) and $A(^2H) = A(^{17}O)$, as for the buried waters W111–W113 in BPTI,[23] we thus obtain $\beta_{COOD}(^2H) = (8.7 \pm 1.2) \times 10^7\ s^{-2}$. It follows from this result that nearly $^2/_3$ of the ^2H dispersion amplitude from the apo protein at pD 6.67 is due to rapidly exchanging COOD deuterons. Inserting $\beta_{COOD}(^2H)$ and $N_{COOD} = 3.24$ (cf. Table 2) into eq 2b, we arrive at $\langle A_{COOD}\chi_{COOD}\rangle = 150 \pm 10$ kHz, representing an average over 1.44 ligand, 1.03 nearby, and 0.77 distant COOD groups.

Using $\chi_{COOD} = 180.3$ kHz, as determined for the side-chain carboxyl deuteron in solid L-glutamic acid hydrochloride,[73] we obtain the class-averaged carboxyl OD bond order parameters $\langle A_{COOD}\rangle = 0.84 \pm 0.06$ for the apo state and $\langle A_{COOD}\rangle = 0.72 \pm 0.08$ for the calcium-loaded state. Since there is some overlap in the COOD populations responsible for these two values, the tendency toward higher order for the ligand carboxyls appears to be significant. These values may be compared with $\langle A_{OD}\rangle = 0.50 \pm 0.05$ as an average over the COOD and OD groups in BPTI,[22] and with $A_{NH} = 0.92 \pm 0.03$ for the majority of main-chain NH bonds (excluding the linker loop and the C-terminus) in calbindin.[42,43]

Surface Hydration. Except for the two calcium-coordinated water molecules and buried surface water(s) (probably only W86), the ca. 300 water molecules that interact directly with the surface of calbindin must have residence times in the subnanosecond range, since they contribute only to the frequency-independent relaxation parameter α. In comparison with the large number of surface waters, the few carboxyl deuterons should make a negligible contribution to $\alpha(^2H)$. This is confirmed by Figure 4b, showing that $\alpha(^2H)$ does not depend significantly on the ionization state of the carboxyl groups. This was also found to be the case for BPTI and ubiquitin.[22]

From Table 1 it is seen that the ratio $\alpha(^{17}O)/\alpha(^2H)$ varies from 89 ± 6 in the apo state to 79 ± 6 in the fully calcium-loaded state. As for BPTI, where this ratio is 83 ± 3,[22] the close agreement with the ratio of the bulk water relaxation rates, $R_{bulk}(^{17}O)/R_{bulk}(^2H) = 77.4 \pm 0.8$,[21,22] shows that, on average, water molecules at the protein surface reorient nearly isotropically. Assuming that $\chi(^{17}O)$ is the same for surface waters as for bulk water, we can estimate the average rotational correlation time, $\langle\tau_S\rangle$, for surface waters from the relation[21]

$$\langle\tau_S\rangle/\tau_{bulk} = 1 + (M_P/M_W)[(1-w)/w](1/N_S)[\alpha(^{17}O)/R_{bulk}(^{17}O)] \quad (5)$$

With $R_{bulk}(^{17}O) = 175\ s^{-1}$,[21] $N_S = 300$, and an average $\alpha(^{17}O)$ from Table 1, we obtain $\langle\tau_S\rangle/\tau_{bulk} = 6.4$, not far from the previously obtained values of 6.2 for BPTI and 5.5 for ubiquitin.[21] With $\tau_{bulk} \approx 3$ ps for D_2O at 27 °C, we thus have $\langle\tau_S\rangle \approx 20$ ps. The small but significant reduction of $\alpha(^{17}O)$ on calcium binding (cf. Figure 4a) and the small increase of τ_R (cf. above) might both be related to the minor Ca^{2+}-induced structural perturbations that have been identified in high-resolution NMR studies.[41–47]

Water Flip Dynamics. The reduction of the ratio $\alpha(^{17}O)/\alpha(^2H)$ on calcium binding, although barely significant, may be a consequence of the flip motion of the calcium-coordinated water molecules. For the few deeply buried waters in, for example, BPTI, the internal motions (mainly subpicosecond librations) are too fast to contribute significantly to α. In contrast, the C_2 flip of the calcium-coordinated water molecules may be much slower than the rotation of surface waters and, hence, could contribute to α. For symmetry reasons, this motion does not contribute to $\alpha(^{17}O)$ (cf. Appendix), but

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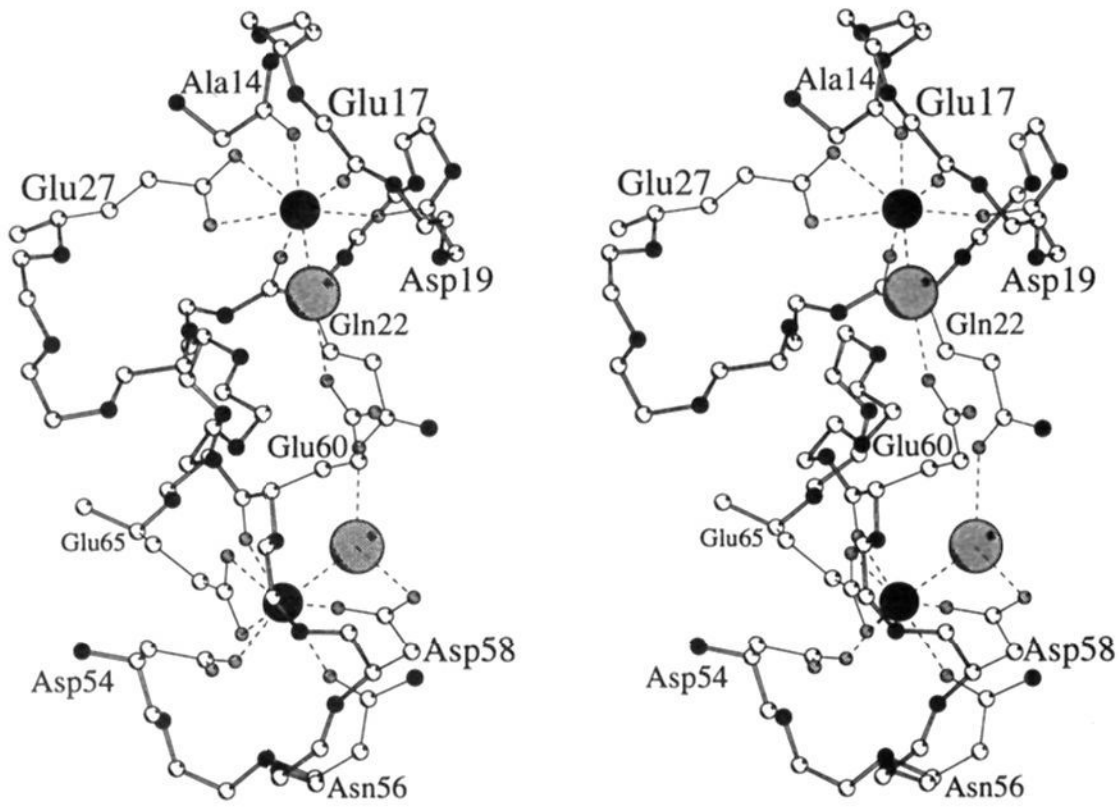

Figure 7. Stereo view of the calcium binding sites (residues 14−27 and 54−65) of calbindin D_{9k}[40] (Protein Data Bank, file 4ICB). Calcium ions are represented by black spheres of radius 0.5 Å and the oxygen atoms of water molecules W78 and W79 by grey spheres of radius 0.7 Å. The small spheres correspond to carbon (white), nitrogen (black), and oxygen (grey) atoms. Main-chain bonds are shown with double lines and side-chain bonds with single lines. Dotted lines link calcium ions with their ligands and the water oxygens with their hydrogen-bonded partners. Site I is at the top and site II at the bottom. The drawing was made with the program Molscript.[88]

adds to $\alpha(^2H)$ a contribution

$$\alpha_{flip}(^2H) = \frac{3\pi^2}{2}\frac{M_W}{M_P}\frac{w}{(1-w)}N_{Ca}\chi^2(1-A_{flip}^2)\tau_{flip} \quad (6)$$

With $A_{flip} = 0.59$ (cf. Figure 6) and α data from Table 1, we find that the variation of $\alpha(^{17}O)/\alpha(^2H)$ with N_{Ca} can be accounted for with a flip correlation time of $\tau_{flip} = 0.3 \pm 0.3$ ns. This rather crude estimate should be regarded as an upper bound. In other words, the essential information about the flip dynamics contained in Figure 4 is not that the relative decrease of $\alpha(^2H)$ is slightly smaller than that of $\alpha(^{17}O)$, but rather that $\alpha(^2H)$ does not increase strongly with N_{Ca}. For example, with $\tau_{flip} = 5$ ns (the upper bound provided by τ_R), $\alpha(^2H)$ would increase from 0.55 s^{-1} in the apo state to 1.2 s^{-1} in the fully loaded state.

Concluding Remarks

We shall now summarize, and put into perspective, the information obtained here about the calcium-coordinated water molecules in calbindin D_{9k}. For this purpose, we reproduce in Figure 7 a part of the crystal structure of calbindin,[40] showing the Ca^{2+} ligands and the hydrogen bonds to the two coordinated water molecules.

The present data demonstrate that the mean residence times of the water molecules that coordinate Ca^{2+} in calbindin D_{9k} are in the range 5 ns to 7 μs at 27 °C. The water residence time in the first coordination shell of metal ions in bulk solution ranges over some 20 orders of magnitude,[74] but that of Ca^{2+} is not accurately known. The widely quoted[74,75] values in the range 1−10 ns for Ca^{2+} were derived, under certain mechanistic assumptions, from measurements of the rate of complex formation between Ca^{2+} and various multidentate chelators.[76,77] The validity of these assumptions has been questioned.[78,79] The most relevant piece of experimental evidence appears to be a quasielastic neutron scattering study, showing that the residence time of metal-coordinated water is much less than 10^{-9} s in 2 and 3 *m* $CaCl_2$ solutions at 25 °C.[78] Water ^1H, ^2H, and ^{17}O relaxation rates from concentrated aqueous solutions of calcium salts,[80−82] although the interpretation is model dependent, are also consistent with a subnanosecond residence time. We must then ask why the residence time of calcium-coordinated water should be longer, possibly by several orders of magnitude, in calbindin than for Ca^{2+} in bulk solution. While the Ca−O distance is closely similar, ca. 2.4 Å, in calbindin[39,40] and in the bulk hydration complex,[83] the interactions within the coordination shell are very different in the two cases. In the crystal structure of calbindin (cf. Figure 7), each water molecule is stabilized by two strong hydrogen bonds: to the carboxyl oxygen of Glu60 (2.64 Å) and to an external water molecule (2.76 Å) in site I, and to the side-chain oxygens of Gln22 (2.62 Å) and Asp58 (2.57 Å) in site II. The 6−10 water molecules (depending on concentration) in the primary shell of the bulk Ca^{2+} aquocomplex[83] are all oriented with their oxygens toward the ion and with their dipole vectors not far from the radial direction.[84,85] In contrast to the stabilizing hydrogen bonds of the calcium-coordinated waters in calbindin, the interactions within the primary coordination shell in the aquocomplex should thus be destabilizing (relative to bulk water), accounting for the shorter residence time.

The qualitatively different response of the ^2H and ^{17}O relaxation dispersions to calcium loading demonstrates that the calcium-coordinated water molecules in calbindin undergo rapid

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C_2 flips, as well as twist librations of considerable amplitude. An examination of the crystal structure in Figure 7 shows that these are, indeed, the most probable modes of internal motion. A (restricted) rotation around the C_2 axis is energetically favored compared to rotations around the two orthogonal axes, since it entails the smallest perturbation of the strong interaction between the Ca^{2+} ion and the water oxygen lone pairs. In fact, if the Ca−O and C_2 vectors are colinear, this interaction is not affected at all by twist librations or C_2 flips. Furthermore, although the two hydrogen bonds per calcium-coordinated water molecule are strong, they involve side chains (and another water molecule) with several degrees of freedom. Superimposed on the fast small-amplitude twist libration that also occurs in solid hydrates, typically with $\tau_{twist} \approx 0.07$ ps,[66] we thus expect a twist libration of larger amplitude, correlated with side-chain motions on the 1−10-ps time scale.

To affect the ^2H dispersion amplitude, the C_2 flip must be fast compared to the reorientation of the protein, i.e., $\tau_{flip} < \tau_R = 5$ ns. Consideration of the (frequency-independent) direct ^2H relaxation contribution from the flip motion shows that τ_{flip} must, in fact, be an order of magnitude shorter than τ_R. In the investigated inorganic crystal hydrates, the activation energy for the C_2 flip ranges from 14 to 68 kJ mol^{-1},[66] corresponding to 9 orders of magnitude variation of τ_{flip} (at room temperature). For comparison with calbindin, the most relevant investigated solid is gypsum, $CaSO_4{\cdot}2H_2O$, where the Ca^{2+} ion coordinates 6 sulfate oxygens (R_{CaO} = 2.38−2.54 Å) and two water molecules (R_{CaO} = 2.38 Å), each of which donates two hydrogen bonds to sulfate oxygens (R_{OO} = 2.82 and 2.90 Å).[59] Apart from the addition of an eighth Ca^{2+} ligand, the environment of the crystal waters in gypsum is thus remarkably similar to that of the calcium-coordinated water molecules in calbindin. The flip rate of the crystal waters in gypsum was determined in an early NMR relaxation study,[62] yielding an unusually low activation energy of 24 kJ mol^{-1} and τ_{flip} = 1.0 ns at 27 °C. This result clearly supports our finding of a fast C_2 flip in calbindin.

On the basis of crystal hydrate data, it has been proposed that the coordination on the lone-pair side of the water oxygen is a major determinant of the activation energy for the C_2 flip;[66] the flip rate is generally much higher when there is only one ligand, as in gypsum and calbindin. In contrast, the deeply buried water molecules in BPTI participate in four strong hydrogen bonds to main-chain atoms (or another buried water) and, hence, should have much slower flip rates. Accordingly, the ^2H and ^{17}O relaxation dispersions from BPTI show no evidence of fast ($\tau_{flip} < \tau_R$) C_2 flips.[20-23]

The present demonstration of the ability of the water NMRD technique to directly observe and dynamically characterize metal-coordinated water molecules in a protein suggests that the same approach can be profitably applied to other diamagnetic metalloproteins. The failure to detect a Zn^{2+}-coordinated water molecule in carbonic anhydrase by single-field ^{17}O transverse relaxation measurements[29] may simply have been a result of using a too low protein concentration. With $A(^{17}O)\chi(^{17}O)$ = 5.6 MHz, as found here, we can estimate the ^{17}O line width contribution from a single long-lived water molecule at a protein concentration of 0.65 mM to 1.3 Hz, which is well within the experimental uncertainty. Although the apo and zinc-loaded states yielded essentially the same relaxation enhancement in a subsequent ^1H NMRD study at higher protein concentration (3 mM),[28] this may be due to the presence of a buried water molecule in the zinc-free binding site.[86]

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Appendix: Generalized Order Parameters for the ^2H and ^{17}O Nuclei in Protein-Bound Water Molecules

For a protein that reorients as a spherical top, the effect of internal motion of water molecules on the relaxation dispersion of the water nuclei ^2H and ^{17}O is fully contained in the generalized order parameter A.[54] This quantity can be defined in terms of the spherical components V_n of the electric field gradient (EFG) tensor as[54]

$$A^2 = (V_0^F)^{-2} \sum_{n=-2}^{2} |\langle V_n^R \rangle|^2 \qquad \text{(A1)}$$

where the angular brackets denote a configurational average over all internal motions that are fast compared to the reorientation of the protein. The superscripts refer to the principal frame (F) of the instantaneous EFG tensor and to the principal frame (R) of the rotational diffusion tensor of the protein. Since A^2 is a rotational invariant, the orientation of the R-frame can be chosen in any convenient way.

By performing the R → F transformation in two steps via a frame (M) fixed in the water molecule, we can express eq A1 on the form

$$A^2 = \sum_n \left| \sum_p \sigma_p S_{np} \right|^2 \qquad \text{(A2)}$$

where the molecular order parameters

$$S_{np} = \langle D_{np}^2(\Omega_{RM}) \rangle \qquad \text{(A3)}$$

are separated from the nucleus-specific EFG coefficients

$$\sigma_p = D_{p0}^2(\Omega_{MF}) + (\eta/\sqrt{6})[D_{p2}^2(\Omega_{MF}) + D_{p-2}^2(\Omega_{MF})] \qquad \text{(A4)}$$

Here $D_{ab}^2(\Omega_{AB})$ is an element of the second-rank Wigner rotation matrix and Ω_{AB} are the Euler angles that effect the A → B transformation.[87] Furthermore, $\eta = \sqrt{6}V_{\pm 2}^F/V_0^F = (V_{xx}^F - V_{yy}^F)/V_{zz}^F$ is the EFG asymmetry parameter as conventionally defined.[53]

With the known orientations of the F frames for ^2H and ^{17}O and the same convention for the M frame as previously adopted (cf. Figure 1 in ref 54), we obtain for ^2H

$$\sigma_0(^2H) = 1 - (1/2)(3 - \eta)\sin^2\alpha \qquad \text{(A5a)}$$

$$\sigma_{\pm 1}(^2H) = (i/2\sqrt{6})(3 - \eta)\sin 2\alpha \qquad \text{(A5b)}$$

$$\sigma_{\pm 2}(^2H) = -(1/\sqrt{6})[\eta + (1/2)(3 - \eta)\sin^2\alpha] \qquad \text{(A5c)}$$

where 2α is the HOH angle in the water molecule. For ^{17}O, we obtain

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$$\sigma_0(^{17}O) = -(1/2)(1 - \eta) \quad \text{(A6a)}$$

$$\sigma_{\pm1}(^{17}O) = 0 \quad \text{(A6b)}$$

$$\sigma_{\pm2}(^{17}O) = (1/2\sqrt{6})(3 + \eta) \quad \text{(A6c)}$$

Explicit expressions for $A(^2H)$ and $A(^{17}O)$ have previously been given for the special case of a uniaxial internal motion (at least C_3 symmetry).[54] In this case, only the $n = 0$ term in eq A2 survives. For highly ordered water molecules buried in proteins, the dominant internal modes should be librational motions around different axes and, possibly, a 180° flip around the C_2 axis of the water molecule. As the symmetry of these motions is lower than C_3, a more general treatment is needed. Here we derive explicit expressions for $A(^2H)$ and $A(^{17}O)$ for symmetric librations around each of three orthogonal axes: plane libration (restricted rotation around the normal to the molecular plane), wag libration (restricted tilting of the molecular plane), and twist libration (restricted rotation around the C_2 axis of the water molecule). In addition, we consider the effect of a C_2 flip (180° rotation around the C_2 axis).

Each of the three librational modes considered has two well-defined limits. In the rigid-lattice limit, i.e., in the absence of internal motion, $S_{np} = \delta_{np}$ and eq A2 reduces to

$$A_0^2 = \sum_n |\sigma_n|^2 = 1 + \eta^2/3 \quad \text{(A7)}$$

For free uniaxial rotation around the respective axis, an evaluation of the order parameters in eq A3 yields with eq A2

$$A^2_{\text{plane}} = (1/4)(\sigma_0 - \sqrt{6}\sigma_2)^2 \quad \text{(A8a)}$$

$$A^2_{\text{wag}} = (1/4)(\sigma_0 + \sqrt{6}\sigma_2)^2 \quad \text{(A8b)}$$

$$A^2_{\text{twist}} = \sigma_0^2 \quad \text{(A8c)}$$

Substitution of the EFG coefficients σ_p from eqs A5 or A6 leads to explicit expressions for $A(^2H)$ and $A(^{17}O)$ in this limiting case.

For a symmetric plane libration of arbitrary amplitude, we obtain

$$A^2_{\text{plane}}(^2H) = A_0^2 - (1/3)(3 - \eta)^2\langle\sin^2\phi_x\rangle\langle\cos^2\phi_x\rangle \quad \text{(A9a)}$$

$$A^2_{\text{plane}}(^{17}O) = A_0^2 - (4/3)\eta^2\langle\sin^2\phi_x\rangle\langle\cos^2\phi_x\rangle \quad \text{(A9b)}$$

where A_0^2 is the rigid-lattice value in eq A7. As expected, A_{plane} does not depend on the molecular angle α. Similarly, we obtain for a wag libration

$$A^2_{\text{wag}}(^2H) = A_0^2 - (1/12)(3 - \eta)^2 \sin^2 2\alpha[1 - \langle\cos\phi_y\rangle] - (1/3)[(3 + \eta) - (3 - \eta)\sin^2\alpha]^2\langle\sin^2\phi_y\rangle\langle\cos^2\phi_y\rangle \quad \text{(A10a)}$$

$$A^2_{\text{wag}}(^{17}O) = A_0^2 - (1/3)(3 - \eta)^2\langle\sin^2\phi_y\rangle\langle\cos^2\phi_y\rangle \quad \text{(A10b)}$$

and for a twist libration

$$A^2_{\text{twist}}(^2H) = A_0^2 - (1/12)(3 - \eta)^2 \sin^2 2\alpha[1 - \langle\cos\phi_z\rangle^2] - (4/3)[\eta + (1/2)(3 - \eta)\sin^2\alpha]^2\langle\sin^2\phi_z\rangle\langle\cos^2\phi_z\rangle \quad \text{(A11a)}$$

$$A^2_{\text{twist}}(^{17}O) = A_0^2 - (1/3)(3 + \eta)^2\langle\sin^2\phi_z\rangle\langle\cos^2\phi_z\rangle \quad \text{(A11b)}$$

The expressions in eqs A9–A11 reduce correctly to the limiting forms, eqs A7 and A8, for $f(\phi) = \delta(\phi)$ and $f(\phi) = 1/(2\pi)$, respectively.

If, in addition to libration, the water molecule undergoes C_2 flips, then the order parameter S_{np} must reflect the acquired C_{2z} symmetry, i.e., S_{np} must vanish for odd p. This has no effect on $A(^{17}O)$, since $\sigma_{\pm1}(^{17}O) = 0$. For ^2H, however, the C_2 flip leads to additional motional averaging. For the plane libration case, eq A9a is modified to

$$A^2_{\text{plane+flip}}(^2H) = A_0^2 - (1/3)(3 - \eta)^2 \times [(1/4)\sin^2 2\alpha + \cos^2 2\alpha\langle\sin^2\phi_x\rangle\langle\cos^2\phi_x\rangle] \quad \text{(A12)}$$

which now depends on the angle α. For the wag and twist cases, the effect of a C_2 flip is simply to cancel $\langle\cos\phi_y\rangle$ and $\langle\cos\phi_z\rangle$ in eqs A10a and A11a, respectively.

Except for $A_{\text{wag}}(^2H)$ and $A_{\text{twist}}(^2H)$, all the generalized order parameters considered here depend on the librational potential of mean torque, $w(\phi)$, or, equivalently, the orientational distribution function $f(\phi)$, via a single parameter, e.g., $\langle\sin^2\phi\rangle$. The two exceptions, however, depend on two independent amplitude parameters. For the purpose of displaying the variation of all order parameters with the libration amplitude (cf. Figure 6), we assume that all orientational distributions are of the form

$$f(\phi) = \begin{cases} 1/(2\phi_0), & \text{if } 0 < |\phi| < \phi_0 \\ 0, & \text{if } \phi_0 < |\phi| < \pi \end{cases} \quad \text{(A13)}$$

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