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A pulsed field gradient NMR study of the aggregation and hydration of parvalbumin

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Abstract

Pulsed field gradient NMR is a convenient alternative to traditional methods for measuring diffusion of biological macromolecules. In the present study, pulsed field gradient NMR was used to study the effects of calcium binding and hydration on carp parvalbumin. Carp parvalbumin is known to undergo large changes in tertiary structure with calcium loading. The diffusion coefficient is a sensitive guide to changes in molecular shape and in the present study the large changes in tertiary structure were clearly reflected in the measured diffusion coefficient upon calcium loading. The (monomeric) calcium-loaded form had a diffusion coefficient of 1.4×10^{-10} m^2 s^{-1} at 298 K, which conforms with the structure being a nearly spherical prolate ellipsoid from X-ray studies. The calcium-free form had a significantly lower diffusion coefficient of 1.1×10^{-10} m^2 s^{-1}. The simplest explanation consistent with the change in diffusion coefficient is that the parvalbumin molecules form dimers upon the removal of Ca^{2+} at the protein concentration studied (1 mM).© 1997 Elsevier Science B.V.

Keywords: Aggregation; Diffusion; Hydration; Parvalbumin; Pulsed field gradient NMR

1. Introduction

Pulsed field gradient (PFG) NMR, due to its non-invasive nature, has been shown to be the method of choice for studying translational diffusion in biological and chemical systems [1]. Although NMR relaxation measurements can be used [2–4], the PFG NMR method is a much more direct means of determining the translational diffusion coefficient. Although the PFG NMR technique was developed in the 1960's, there have not been many papers dealing with biopolymers until recently. In 1968, Moll [5] showed that PFG NMR is a very sensitive tool for observing helix–coil transitions. More recently, PFG NMR has been shown to be a sensitive probe of the size and shape of amylopectin [6], protein hydration [7] and aggregation [8,9] and for providing independent estimates of rotational diffusion coefficients for use in NMR relaxation studies of protein backbone dynamics [10]. The diffusion coefficient of a species can be related to the shape and molecular weight ($\sim \alpha$ volume) of a species. However, care must be taken in relating the observed diffusion coefficient to the underlying molecular occurrences, especially to

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the effects of hydration and distributions of diffusing species (if any).

Carp parvalbumin (CPA) (pI 3.95; MW ~ 12000) is one of a highly homologous class of acidic and highly soluble calcium-binding proteins in the sarcoplasm of vertebrate skeletal muscle. Due to its high concentration (0.5–2 mM) and large binding constant for calcium ($K_{Ca} \sim 2 \times 10^8$), it is able to bind all of the calcium in the sarcoplasm where it is thought that the protein plays a regulatory role. The X-ray crystallographic structure of calcium-loaded parvalbumin has been solved [11–13]. It was found that the molecule is a relatively rigid nearly spherical prolate ellipsoid with a well-defined hydrophobic core that occupies about one-seventh of its total volume. The volume of the entire molecule is 16900 Å^3. It was found that there is a shell about 2.7 Å thick containing atoms, exclusive of protons that would be accessible to the solvent in the absence of crevices. A van der Waal's radii study of crystallographic data indicates that 42% of the crystal volume is occupied by solvent [12].

The hydrophobic core is composed of six α-helical loops (A–F) and X-ray crystallographic studies have shown that parvalbumin binds two calcium ions between helices C and D and helices E and F. One calcium ion (denoted the CD calcium) is completely surrounded by the protein ligands and so is not accessible to the solvent, whilst the other ion (denoted the EF calcium) has a water molecule in the primary hydration sphere [13]. For brevity, we denote the calcium-depleted, singly and doubly bound states of parvalbumin by CPA–Ca_0, CPA–Ca_1 and CPA–Ca_2, respectively.

To date, there have been a number NMR studies on parvalbumin; for reviews see Refs. [14–16]. ^{1}H and ^{13}C NMR measurements have revealed that the phenylalanine residues in the hydrophobic core have highly mobile side chains [17]. ^{1}H NMR studies have shown that the folding of the polypeptide chain to form the compact tertiary structure of CPA–Ca_2 is accompanied by very large upfield shifts of both aliphatic and aromatic resonances. The large upfield shifts are thought to result from ring current shifts produced by the aromatic rings of the ten phenylalanine residues. It has been inferred from the upfield shifts that the characteristic packing of most of the phenylalanine side-chains within the internal core is maintained in solution [14]. When the protein is depleted of calcium, not only are the very large upfield shifts lost but the major proportion of the narrow resonances in the ^{1}H NMR spectrum disappear as well. These changes have been taken to indicate the (nearly) total disorganization of the hydrophobic core. The results of other forms of spectroscopy (CD, ORD and SANS) indicate that the polypeptide is still mainly organized into α-helical segments and thus it can be inferred that the organization of the hydrophobic core (tertiary structure) and α-helices (secondary structure) are largely independent. The presence of Ca^{2+} or other polyvalent ions is necessary to maintain the integrity of the hydrophobic core and it has been found that the tertiary structure varies reversibly between the (compact) CPA–Ca_2 to the (expanded) CPA–Ca_0 form [18].

Analysis of the ^{13}C NMR longitudinal relaxation time for α-carbons ($T_1 \approx 55$ ms) and the nuclear Overhauser effect (NOE) values (≈ 1.2) for α-carbons in a 15 mM sample of CPA–Ca_2 at 295 K yield a value of about 12 ns for the reorientational correlation time (τ_c) of the parvalbumin molecule [19,20]. This compares favourably with the value of 12 ns obtained from light-scattering measurements at 294 K [19], but unfavourably with those from ^{43}Ca NMR relaxation measurements at 296 K using a protein concentration of 5.6 mM which resulted in a τ_c value of 4 ± 1 ns [21]. However, the value derived from the ^{43}Ca relaxation measurements is consistent with the value of 3–4 ns obtained using the Stokes–Einstein equation (see below) and assuming the protein to be a sphere with a diameter of 30–32 Å [21,22], and the discrepancy with the ^{13}C NMR-derived value may reflect large differences in the experimental protein concentration [20].

In the current work, we use PFG NMR to characterize the CPA–Ca_0 and CPA–Ca_2 forms of carp parvalbumin. PFG NMR diffusion measurements of the two forms were undertaken at 298 K and the resulting diffusion measurements were analysed to infer the likely molecular shape of the two forms. In particular, the difference in diffusion coefficient between the two forms was used to discriminate whether the large changes that occur in the proton spectrum upon the removal of calcium result from changes in the tertiary structure or aggregation.

2. Theory

2.1. The PFG experiment

Various sequences exist for measuring the translational diffusion coefficient using PFG NMR, e.g. see Ref. [1]. The simplest sequence is a modified Hahn spin-echo sequence where the loss of signal due to relaxation is proportional to $\exp(-2\tau/T_2)$, where 2τ is the total echo time and T_2 is the transverse relaxation time. However, in the case of macromolecules the condition $T_1 \gg T_2$ (T_1 is the longitudinal relaxation time) normally holds and it can be advantageous to use the stimulated echo pulse sequence [23] as depicted in Fig. 1. This sequence is well-suited to the study of macromolecular diffusion since the protein magnetization is stored along the z-axis during the major part, i.e. τ_2, of the Δ period. In the case of isotropic free diffusion, the attenuation of the echo amplitude is given by [24]

$$E = \frac{M(g)}{M(0)} = \exp\left(-\gamma^2 g^2 D\delta^2(\Delta - \delta/3)\right) \qquad (1)$$

where D is the translational diffusion coefficient, γ is the gyromagnetic ratio, g is the magnitude and δ is the duration of the gradient pulses, and Δ is the separation between the leading edges of the pulses. Thus, after calibration of the spectrometer, the only unknown is D and so by performing a series of experiments where either g, δ or Δ are varied, an estimate for D can be straightforwardly determined. In the current series of experiments, we chose to vary g and thus a plot of $\ln(E)$ versus g^2 yields a straight line with a slope of $-\gamma^2 D\delta^2(\Delta - \delta/3)$.

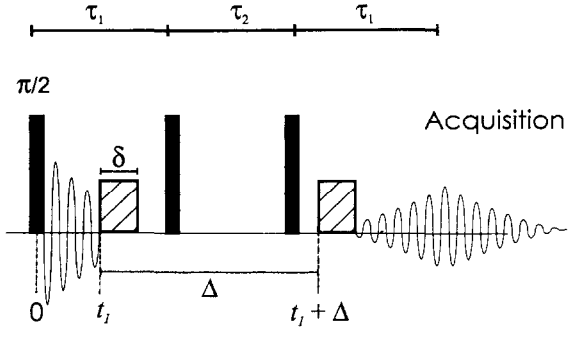

Fig. 1. The stimulated echo pulse sequence. The magnetization is first tipped into the $x-y$ plane by the first $\pi/2$ radio-frequency pulse. The first, i.e. dephasing, gradient pulse of duration δ is then applied and at the end of the period τ_1 the magnetization is rotated by another radio-frequency pulse so that it lies along the z-axis. During τ_2, the relaxation of the magnetization is governed by T_1. At the end of the period τ_2, the magnetization is again tipped into the $x-y$ plane by the third radio-frequency pulse and the second, i.e. rephasing, gradient pulse is applied and the stimulated echo forms. The second part of the echo is collected and used as the free induction decay. To reduce the phase cycling requirements, a homospoil pulse (not shown) was inserted into the delay τ_2.

If there is a distribution of diffusing species then Eq. (1) is no longer applicable and this situation is further complicated if the different species in the distribution have a range of relaxation times [25] since the contributions from the diffusing species will be controlled by their relaxation times as well as their relative populations. For example, if the diffusing species exists in n different aggregation states, i.e. monomer, dimer ... n-mer, then the observed echo intensity from the stimulated echo pulse sequence would be given by

$$M(g) = \sum_n M_{0,n}\exp(-2\tau_2/T_{2,n})\exp(-\tau_1/T_{1,n}) \times \exp\left(-\gamma^2 g^2 D_n \delta^2(\Delta - \delta/3)\right) \qquad (2)$$

where the subscript n refers to the aggregation state of the contributing species and M_0 denotes the equilibrium magnetization. If the relaxation time difference can be neglected then the data can be normalized as in Eq. (1). The situation can be further simplified if a particular type of diffusion coefficient distribution can be assumed [1,26]. If there is a difference in the transverse relaxation times between the different species, diffusion measurements can be performed with different values of τ_1 while keeping Δ constant to probe the different contributions [25,27].

2.2. Macromolecular shape and diffusion

The translational diffusion coefficient of a macromolecule is related to the friction coefficient (f) of the molecule by the Stokes–Einstein equation, e.g. see Refs. [28,29]

$$D = \frac{kT}{f} \qquad (3)$$

where k is the Boltzmann constant and T is temperature. The friction coefficient of a protein is deter-

mined by its overall dimensions, hydration and the rugosity of the surface exposed to the solvent, i.e. water in the present case [30]. Protein atoms that are not exposed to the solvent and solvent molecules trapped inside the protein do not contribute to the friction coefficient.

The friction coefficient is particularly simple for the case of a sphere and is given by (Stokes law)

$$f_0 = 6\pi\eta R \tag{4}$$

where η is the solvent viscosity and R is the radius of the macromolecule. For the case of a prolate ellipsoid, Eq. (4) becomes more complicated and is given by

$$f = 6\pi\eta R \frac{\sqrt{1-(b/a)^2}}{(b/a)^{2/3}\ln\left(\dfrac{1+\sqrt{1-(b/a)^2}}{b/a}\right)} \tag{5}$$

where a and b are the dimensions of the major and minor axes, respectively, and R is the radius of a sphere of equal volume. Friction coefficients have also been derived for more complicated geometries, e.g. Refs. [31–33].

Including the effects of hydration, the effective hydrodynamic radius of the macromolecule is given by

$$R = \sqrt[3]{\frac{3M}{4\pi N}\left[\bar{\nu}_{\text{solute}} + g_{\text{sol}}\bar{\nu}_{\text{solvent}}\right]} \tag{6}$$

where M is the molecular weight, N is Avogadro's number, $\bar{\nu}_{\text{solute}}$ is the partial specific volume of the solute, i.e. protein, $\bar{\nu}_{\text{solvent}}$ is the partial specific volume of the solvent, and g_{sol} is the weight ratio of the solvent to solute. Eqs. (3)–(6) can be rearranged to give

$$M = \left(\frac{kT}{6\pi\eta FD}\right)^3\left(\frac{4\pi N}{3\left[\bar{\nu}_{\text{solute}} + g_{\text{sol}}\bar{\nu}_{\text{solvent}}\right]}\right) \tag{7}$$

where F $(=f/f_0)$ is the Perrin factor.

It is known that the microviscosity of macromolecular solutions is only weakly influenced by the macromolecule component at low macromolecular concentrations [2]. Thus in the present work we have taken the solution viscosity of the protein solution to be equal to that of water at 298 K (8.904×10^{-4} kg m^{-1} s^{-1} [34]). From the molecular volume determined by X-ray diffraction, i.e. 16900 Å^3, the partial specific volume for the protein, i.e. $\bar{\nu}_{\text{solute}}$) was calculated to be 0.85×10^{-3} m^3 kg^{-1}; however, this figure appears to be too high with respect to the partial specific volumes obtained for similar proteins [28,33]. Consequently we have assumed a value of 0.73×10^{-3} m^3 kg^{-1} in the calculations.

3. Experimental

Ca^{2+}-free parvalbumin pI 3.95 (lyophilized) was prepared from carp (*Cyprinus carpio*) skeletal white muscle by the method described previously [35–37]. Ca^{2+} bound to parvalbumin molecules was removed by treatment with trichloroacetic acid [38]. NMR samples were prepared by dissolving parvalbumin in H_2O:D_2O = 95:5 containing 100 mM KCl to give a concentration of 1 mM and the pH was adjusted to 6.5 using NaOH or HCl.

^{1}H PFG NMR experiments were performed at 500 MHz using a Bruker DMX 500 equipped with a 5 mm triple resonance probe with 3-axis-shielded magnetic field gradients. In the experiment only the z-gradient was employed and its strength was first calibrated using the known diffusion coefficient of water [39]. All measurements were performed at 298 K. The temperature in the NMR probe was calibrated using methanol [40].

Samples ($\sim$0.1 ml) for the PFG experiments were placed into susceptibility-matched microtubes (Cat. BMS-005; Shigemi, Tokyo) and the position of the NMR tube was adjusted so that the sample was in the most linear region of the magnetic field gradient. The Hahn spin-echo pulse sequence was used to measure the T_2 of the protein samples. The stimulated echo pulse sequence was used to measure the translational diffusion coefficients since for macromolecules $T_1 \gg T_2$. Typical acquisition parameters were τ_1 = 15 ms, τ_2 = 62 ms, Δ = 90 ms and δ = 3 ms, and the gradient strength was varied up to a maximum of 0.55 Tm^{-1}. In all cases, a spectral width of 7 kHz was digitized into 16 K data points. Each spectrum is the average of 80 transients. A recycle delay sufficient to allow for full relaxation, i.e. $> 5 \times T_1$, was allowed between each transient. Different values of τ_1 and Δ were used to assess

whether the parvalbumin in the calcium-loaded or unloaded state was present as a single species or as a distribution, or whether the measurements were affected by exchange.

4. Results

Due to signal-to-noise limitations, the diffusion of parvalbumin was studied by integrating over the 0.5–1.0 ppm section of the aliphatic region of the spectrum. The high field end of the proton spectrum was chosen so as to exclude complications with any exchangeable groups, i.e. –NH or –OH. The average T_2 of this region was 25 ms in the calcium-loaded form and 27 ms in the calcium-depleted form. Some typical PFG spectra of the calcium-loaded and -depleted parvalbumin for the case of $\Delta = 90$ ms are presented in Fig. 2. The corresponding analysis of the data is presented in Fig. 3.

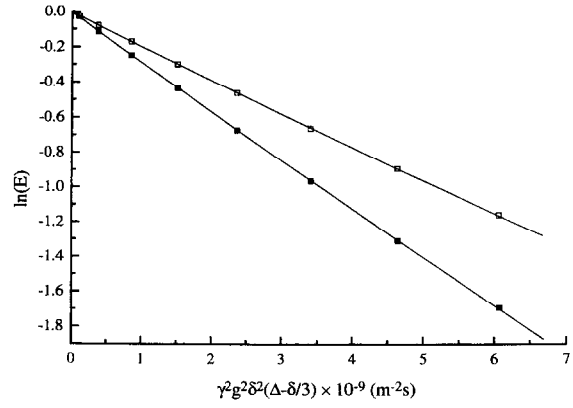

Fig. 3. A plot of the $\ln(E)$ versus $\gamma^2 g^2 \delta^2(\Delta - \delta/3)$ for parvalbumin in the CPA–Ca_2 (♦) and CPA–Ca_0 forms (◇). The experimental parameters are given in the caption to Fig. 2. Specifically, the data are for the non-exchangeable aliphatic resonances in the region 0.5 to 1 ppm. The solid lines are the results of linear regression onto each data set. The diffusion coefficients of the calcium bound and free protein are 1.4×10^{-10} m^2 s^{-1} and 1.1×10^{-10} m^2 s^{-1}, respectively.

4.1. The calcium-loaded (CPA–Ca_2)

The calcium-loaded form is known to be monomeric. The results of our PFG experiments at 298 K give the diffusion coefficient to be about 1.4×10^{-10} m^2 s^{-1}. Using Eq. (3) we calculate the friction coefficient (f) to be 2.9×10^{-11} J m^{-2} s,

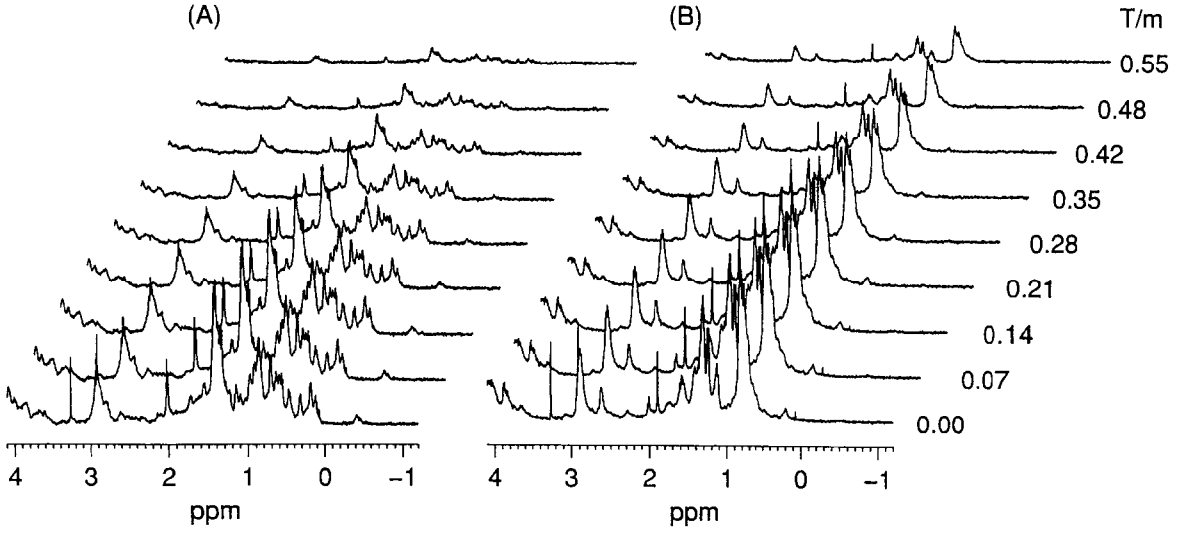

Fig. 2. ^{1}H PFG NMR spectra (aliphatic region) of parvalbumin in the (A) presence and (B) absence of calcium versus the magnitude of the magnetic field gradient pulses (g) at 298 K. The experimental parameters used were $\tau_1 = 15$ ms, $\tau_2 = 62$ ms, $\delta = 3$ ms and $\Delta = 90$ ms with g ranging in equal increments from 0 to 0.55 Tm^{-1}. The spectra are presented with a line broadening of 1 Hz to improve the apparent signal-to-noise ratio. The translational diffusion coefficient of the calcium bound and calcium depleted are quite different, as is evident by the large difference in the attenuation between the spectra acquired with the highest gradient strength. Also clearly apparent is the loss of fine structure in the spectra of the calcium-depleted parvalbumin. The corresponding diffusion plots are given in Fig. 3.

whereas the friction coefficient for a sphere of equivalent volume, i.e., $g_{sol} = 0$, should, using Eq. (4), have a friction coefficient (f_0) of 2.7×10^{-11} J m^{-2} s using the radius derived from the crystallographic data, i.e. $R = 15.9$ Å. Thus, the maximum asymmetry is given by $F\ (= f/f_0) = 1.10$, which according to Eq. (5) would correspond to $a/b = 2.9$. If, however, we use a value for the radius of 15 Å derived from Eq. (6) using a value of $\bar{\nu}_{solute} = 0.73 \times 10^{-3}$ m^3 kg^{-1} and setting $g_{sol} = 0$, then using Eq. (4), f_0 becomes 2.5×10^{-11} J m^{-2} s. This would result in the maximum asymmetry being given by $F = 1.16$, which corresponds to $a/b = 3.7$. At the other extreme, if we consider the protein to be a perfect sphere and using Eqs. (3), (4) and (6), the maximum value of g_{sol} was determined to be 0.4 g H_2O per g of protein or 0.28 g H_2O per g of protein if $\bar{\nu}_{solute}$ is derived from the crystallographic volume. If a value of 0.35 g H_2O per g of protein is used for g_{sol} (see Section 5) and $\bar{\nu}_{solute} = 0.73 \times 10^{-3}$ m^3 kg^{-1}, then the ratio a/b is reduced to 1.5 and the effective radius is given by Eq. (6) as 17.3 Å.

4.2. The calcium-depleted form ($CPA–Ca_0$)

The results of our PFG experiments at 298 K give the diffusion coefficient to be about 1.1×10^{-10} m^2 s^{-1}. Assuming that this diffusion coefficient is due to a monodisperse protein aggregate, then using Eq. (3), we calculate the friction coefficient (f) to be 3.7×10^{-11} J m^{-2} s. If we further assume that $g_{sol} = 0.35$ g H_2O per g of protein and that $a/b = 1.5$ (as for the monomeric case), then the apparent molecular weight of the calcium-depleted form is about 25 000, which is roughly what would be expected for a parvalbumin dimer. This corresponds to an effective radius of 22.0 Å.

5. Discussion

PFG NMR is a very convenient alternative to traditional methods such as quasi-electric light scattering and sedimentation analysis for studying the self-association of proteins. Furthermore, the concentration range used in the experiments is exactly that used in NMR structural determination, whereas the traditional methods of studying protein aggregation are generally carried out at much lower concentrations. The results on protein aggregation obtained from PFG NMR measurements provide complementary information to that obtained from the structural studies [9]. Knowing the degree of self-association of a protein is a prerequisite for NMR structural studies and it is particularly convenient to be able to use an NMR method on the same sample as that used for the structural studies, especially when few laboratories have access to other methods, e.g. an analytical ultracentrifuge, for studying protein aggregation. It also allows the study of the aggregation state of the protein, such as at a certain pH or in the presence of ions, which would not be possible by other means.

Measuring the diffusion coefficient using PFG NMR is a very direct means of studying the apparent molecular weight and shape of a protein, e.g. Eqs. (3)–(7), and is much more straightforward than using the NMR relaxation time. In the present study, the measured protein diffusion coefficients measured using PFG NMR changed quite significantly depending on the degree of calcium loading, yet the average T_2 over the non-exchangeable protons did not change significantly with calcium loading. In the absence of any interfering mechanisms, the NMR relaxation times of the aliphatic protons (predominantly on amino acid side chains) depend not only on the motion of the whole molecule but also on the local motion of the amino acid side chains themselves. It is very likely that these local side-chain motions, especially of the amino acid residues that still face the solvent, are largely unchanged upon aggregation. Furthermore, the shape of, for example, a dimer is unlikely to be equal to that of an enlarged monomer and thus the correlation times that describe the reorientation (and ultimately the NMR relaxation time) of the monomer and dimer will not be directly connected. Similarly, we measured an average T_2 over the non-exchangeable protons; upon aggregation the weighting of this averaging process will be changed as some formerly exposed residues will now be shielded from the solvent and thus be more immobile. Thus, even without outside interference, many assumptions need to be made in using the relaxation time approach in studying protein aggregation. It should be noted that the presence of paramagnetic ions, e.g. dissolved oxygen and paramagnetic ions bound to the protein, even at very low concentra-

tions, can strongly influence relaxation measurements. In the present study, it is not improbable that small quantities of paramagnetic ions could have been inadvertently introduced into the sample upon addition of the Ca^{2+} ions. Generally in relaxation measurements, it is very difficult to separate intermolecular sources of relaxation from the (information providing) intramolecular sources [4]. PFG NMR, on the other hand, is only sensitive to the size and shape of the molecule and the solution viscosity.

To minimize complications arising from cross-relaxation and exchange between the water and protein protons, only the resonances of aliphatic, i.e. non-exchangeable, protons were used to probe the protein diffusion coefficients [41]. In agreement with this, the measured diffusion coefficients were, within experimental error, independent of the observation time, i.e. Δ (data not shown). In order to test for the effects of polydispersity in the sample, relaxation weighting can be added to the measured diffusion data by replacing the first $\pi/2$ pulse in the stimulated echo sequence by $\pi/2$-τ-π-τ [25]. However, since with the present experimental equipment the gradient-induced eddy current effects have completely dissipated after a delay of 100 μs (results not shown), we adopted the simpler approach of directly altering τ_1 in the stimulated echo sequence, thus avoiding further spectral complications due to the effects of J-modulation during the additional echo. However, within experimental error, there was no τ_1 dependence of the measured diffusion coefficient (data not shown). This is probably not surprising when it is considered that there was no significant difference between the average T_2 values for the calcium-loaded and unloaded forms of parvalbumin. Thus, we were justified in neglecting the effects of any T_2 weighting (see Eq. (2)) on the measured diffusion coefficients. Conversely, the presence of polydispersity could not be probed on the basis of T_2 weighting.

Ahlström and coworkers have investigated interfacial water structure and dynamics in parvalbumin solutions [42,43]. Although their simulations were performed under quite different "experimental" conditions, i.e. protein concentration and temperature, the simulations revealed the importance played by the water in maintaining the protein structure [42]. In determining the degree of hydration, a cogent estimate of the partial specific volume must be used in the calculations. It is well known that the molecular volume is generally of the order of 25% smaller than that obtained from the partial specific volume. The partial specific volumes for typical proteins are in the range of $0.70–0.75 \times 10^{-6}$ m^3 kg^{-1} [28,33]. A likely reason for the discrepancy between the actual molecular volume and that calculated from the partial specific volume is the inclusion of water molecules within the protein structure. Thus, if the actual molecular volumes are used the degree of hydration determined will be underestimated. The inclusion of the effects of hydration and the Perrin factor on the diffusion coefficient is a difficult problem. Due to practical and theoretical limitations we are restricted to considering the hydration water as being a uniform hydration shell. Zhou [33] has shown that for typical globular proteins the inclusion of a hydration shell around 0.9 Å thick gave good agreement between calculated and experimentally derived diffusion coefficients. A hydration shell of this thickness corresponds to $g_{sol} = 0.3–0.4$ g H_2O per g of protein. A value of 0.34 corresponds to a hydration shell with a thickness of about one water molecule.

From analysing the data for the CPA–Ca_2 form and assuming the reasonable hydration value of 0.35 g H_2O per g of protein, we obtain the ratio a/b of 1.5 which is in good agreement with the near-spherical nature of parvalbumin as noted by X-ray diffraction. Due to the near-spherical nature of the CPA–Ca_2 form, it is not unreasonable to model the reorientational correlation time of the protein by a single correlation time, i.e. τ_c. Setting $R = 17.3$ Å and using the Debye equation [44], we calculate τ_c to be 4.7 ns which is in good agreement with the ^{43}Ca NMR relaxation measurements [21]. Our estimate is considerably less than that derived from ^{13}C measurements, i.e. 12 ns, [19,20]; however, those measurements were performed at a much higher protein concentration, i.e. 15 mM.

In order to discuss the likely cause of the decrease in the observed diffusion coefficient upon the removal of Ca^{2+}, we need to consider the difference between aggregation and changes in tertiary structure upon the removal of Ca^{2+}. From simulations using Eq. (5), it can be shown that the Perrin factor is not very sensitive to the degree of non-sphericity of the protein; for example a prolate ellipsoid with the

rather extreme ratio of $a/b = 5$ will have a Perrin factor of only 1.25, which is insufficient to account for the change in the observed diffusion coefficient upon the removal of Ca^{2+}. Similarly, g_{sol} can be varied to a large degree without drastic effects on the observed molecular weight. The ratio of the observed diffusion coefficients for the calcium-loaded and -depleted forms of parvalbumin, (i.e., $D_{CPA-Ca_2}:D_{CPA-Ca_0} = 0.79$) is very close to the predicted value of 0.75 estimated by approximating the monomer–monomer interaction as a hard-sphere molecular interaction [30]. Thus, the simplest explanation that we can realize for our experimental results is that removal of Ca^{2+} results in the dimerization of parvalbumin molecules under the present experimental conditions. From the effective radius of 22.0 Å, we calculate the reorientational correlation time of the calcium-free form to be 9.6 ns.

6. Conclusions

The results of the present work have shown that PFG NMR diffusion measurements are a convenient alternative to traditional means of measuring the diffusion coefficient of proteins in solution. Analysis of the resulting diffusion data can then provide a wealth of information about the shape and aggregation state(s) of the diffusing species. The measurements also provide information which is complementary to NMR structural studies. The simplest explanations for the observed diffusion coefficients for the parvalbumin system studied is that the fully calcium-loaded form is monomeric while the calcium-free form is dimeric.

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