have applied the R value method to a variety of 5-membered carbocycles and heterocycles; the results, while indicating trends, were not interpretable quantitatively. Lambert et al. have pointed out that the evaluation of an average dihedral angle from the $^3J_{cis}$ coupling constants was more successful and suggest that this may be due to rehybridization of the carbons giving angles between trans protons in the planar state other than 120°.

Kuo and Gibbons[11] have used a double Karplus curve-ring closure method to ascertain values for χ^1, χ^2, and χ^3 in the Pro[5] in tyrocidine A in $(CD_3)_2SO$. It is interesting to compare their results with those obtained by applying the analysis developed in this paper. For χ^1, Kuo and Gibbons reported +30°, while our method gives $\hat{\chi}^1 = 28°$, $\tilde{\chi}^1 = +26°$. The near equality of $\hat{\chi}^1$ and $\tilde{\chi}^1$ implies little torsion about the $\alpha\beta$ C-C bond, and the agreement between the two methods is excellent. For χ^2, Kuo and Gibbons obtain −34°, while we obtain $\hat{\chi}^2 = 31°$, $\tilde{\chi}^2 = +21°$. For χ^3, Kuo and Gibbons obtain +30°, while we get $\hat{\chi}^3 = 26°$, $\tilde{\chi}^3 = +21°$. In each case, therefore, we agree well with the estimate of average nonplanarity but differ in that we deduced a significant population of conformer with lesser or opposite twist.

Jones, Kuo, and Gibbons[12] have performed a partial analysis of the proton spectrum of gramicidin S in CD_3OD and have deduced $\langle\chi^1\rangle = 25°$. From the reported values of J_c and J_t we obtain $\hat{\chi}^1 = 25°$, $\tilde{\chi}^1 = +23°$, in complete agreement.

Analyses of L-proline itself have been reported by Ellenberger et al.[28] and by Pogliani et al.[29] Anteunis et al.[30] have synthesized *trans*-2,3-dideuterio-L-proline, and their analysis is consistent with those reported by the other two groups. For L-Pro, using Ellenberger's values for the coupling constants, we obtain $\hat{\chi}^1 = 22°$, $\tilde{\chi}^1 = +4°$, $\hat{\chi}^2 = 24°$, $\tilde{\chi}^2 = +1°$, and $\hat{\chi}^3 = 28°$, $\tilde{\chi}^3 = 0°$, confirming that in free proline all puckering modes are active and that there is little A-B preference.

Acknowledgment. The NMR Facility for Biomedical Studies at Carnegie-Mellon is supported by grants from the Division of Research Resources, National Institutes of Health (No. RR00292), and from the National Science Foundation (No. PCM 7815113-A01). In addition, this research has been supported by Grants GM 27616 (L.M.G.) and AM-16532 (A.A.B.) from the National Institutes of Health. Acknowledgement is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this work (L.M.G.). A.C.B. is an Upjohn Graduate Fellow. We appreciate the careful review of the manuscript by Dr. Herman R. Wyssbrod.

Registry No. I, 75929-66-7; II, 66143-40-6.

(28) Ellenberger, M.; Pogliani, L.; Haüser, K.; Volat, J. *Chem. Phys. Lett.* **1974**, *27*, 419.
(29) Pogliani, L.; Ellenberger, M.; Valat, J.; Bellocq, A. M. *Int. J. Pept. Protein Res.* **1975**, *7*, 345.
(30) Anteunis, M. J. O.; Borremans, F. A. M.; Becu, C.; Sleeckx, J. *Int. J. Pept. Protein Res.* **1979**, *14*, 445.

Direct Observation of the ^{43}Ca NMR Signals from Ca^{2+} Ions Bound to Proteins

Thomas Andersson,* Torbjörn Drakenberg, Sture Forsén, Eva Thulin, and Marianne Swärd

Contribution from the Departments of Physical Chemistry 1 and 2, Chemical Center, University of Lund, S-220 07 Lund 7, Sweden. *Received June 5, 1981*

Abstract: The ^{43}Ca NMR signals from Ca^{2+} ions bound to the Ca^{2+} binding proteins parvalbumin, troponin C, and calmodulin have been observed. The observation was made possible through the combined use of isotopically enriched $^{43}Ca^{2+}$, FT techniques, high magnetic fields, and a solenoid type of probe design. Measurements of the apparent longitudinal relaxation rate, R_1, and the transverse relaxation rate, R_2, provide values of both the quadrupole coupling constant and the correlation time. The magnitude of the calculated correlation times is in good agreement with the rotational correlation time for the entire protein molecules, indicating the Ca^{2+}-binding sites to have a comparatively rigid structure.

Much of the pioneering works on the NMR of the ^{43}Ca nucleus created an undue pessimism about the usefulness of this method for the study of Ca^{2+}-macromolecule interactions.[1] Many of these investigations were performed at the time when Fourier transform NMR spectrometers and cryomagnets were not available. During the last few years several studies have shown that ^{43}Ca NMR can give interesting information about Ca^{2+} binding to proteins even at millimolar concentrations.[2,3] These studies were performed on systems with relatively fast chemical exchange of the Ca^{2+} ion between the free and protein-bound states; i.e., the observed ^{43}Ca signal is an average signal sensitive to the exchange rate. The application of this method is limited to proteins with Ca^{2+} affinities less than ca. 10^5-10^6 M^{-1}.

In the work presented here we show that the direct observation of the ^{43}Ca NMR signal of the protein-bound Ca^{2+} ions is in some cases possible and that relevant physical and biological information can be obtained from such studies. As examples of this approach we have used three Ca^{2+}-binding proteins: carp muscle parvalbumin, pI = 4.25, rabbit skeletal muscle troponin C, and bovine testes calmodulin.

Experimental Section

Bovine testes calmodulin (CaM) and rabbit skeletal muscle troponin C (TnC) were prepared as described in ref 4 and ref 3, respectively. Carp muscle parvalbumin component, pI = 4.25 (PA), was generously supplied by Professor J. Parello, Montpellier, France.

Ca^{2+}-free TnC and CaM were prepared by passing an aqueous solution of the proteins through a column of Chelex-100. The solutions were then lyophilized and at the time of use dissolved in doubly distilled water. The Ca^{2+} content of TnC and CaM after this treatment, as measured by atomic absorption spectrophotometry, was ≤0.2 mol of Ca^{2+}/mol of protein. For PA, this procedure did not give a sufficient degree of deionization due to the higher affinity of PA for Ca^{2+}. Consequently, Ca^{2+} was replaced by Cd^{2+} ions by simply adding an excess of Cd^{2+} ions to the protein (PA binds Cd^{2+} more strongly than Ca^{2+}), and the excess

(1) Lindman, B.; Forsén, S. In "NMR and the Periodic Table"; Harris, R. K., Mann, B. E., Eds.; Academic Press: London, 1978.
(2) Andersson, T.; Drakenberg, T.; Forsén, S.; Wieloch, T.; Lindström, M. *FEBS Lett.* **1981**, *123*, 115.
(3) Andersson, T.; Drakenberg, T.; Forsén, S.; Thulin, E. *FEBS Lett.* **1981**, *125*, 39.
(4) Forsén, S.; Thulin, E.; Drakenberg, T.; Krebs, J.; Seamon, K. *FEBS Lett.* **1980**, *117*, 189.

0002-7863/82/1504-0576$01.25/0

Table I. Relaxation Data for a Spin $^7/_2$ Nucleus at $\omega\tau_c = 1.5$, $\omega = 1.078 \times 10^8$ s^{-1}, and $\chi = 1$ MHz

relative amplitude coeff			apparent relaxation rate,[b] s^{-1}	
$t_d = 0$	$t_d = 300$ μs	relaxation rate,[a] s^{-1}	$t_d = 0$	$t_d = 300$ μs
$C_{2,1} = 0.832$	$C_{2,1} = 0.912$	$R_{2,1} = 2.12 \times 10^3$	$R_2 = 2.22 \times 10^3$	$R_2 = 2.17 \times 10^3$
$C_{2,2} = 0.083$	$C_{2,2} = 0.068$	$R_{2,2} = 3.09 \times 10^3$		
$C_{2,3} = 0.079$	$C_{2,3} = 0.019$	$R_{2,3} = 7.13 \times 10^3$		
$C_{2,4} = 0.006$	$C_{2,4} = 0.0002$	$R_{2,4} = 12.98 \times 10^3$		

[a] The relative amplitudes precisely after the pulse ($t_d = 0$) and after the instrument dead time ($t_d = 300$ μs) described in ref 8. [b] The apparent relaxation rates obtained from the line width at half-height of the actual superposition of four lorentzian lines at $t_d = 0$ and $t_d = 300$ μs.

salt was removed by dialysis. The protein solution was then deionized on the Chelex-100 column, which has a much higher affinity for Cd^{2+} ions than for Ca^{2+} ions, resulting in ~0.2 mol of Cd^{2+}/mol of protein.

An aqueous 0.115 M $CaCl_2$ solution was prepared by dissolving $CaCO_3$ (60% isotopically enriched in ^{43}Ca, Oak Ridge National Laboratory, Oak Ridge, TN) in 1 M HCl. The solution was neutralized to a final pH of 7.0 by adding 1 M NaOH.

The ^{43}Ca NMR spectra were recorded at 17.16 MHz by using a homemade Fourier transform spectrometer equipped with an Oxford Instruments 6-T wide-bore magnet (room temperature; bore, 89 mm). In order to increase the signal to noise ratio, we constructed the probe with a horizontal sample orientation so that the higher performance solenoid coils could be used.[5] By this procedure the signal to noise ratio was found to increase by a factor of 2.5 (corresponding to a gain in time by a factor of 6) compared to the conventional vertical Helmholtz coil design. The length for 90° pulses varied between 50 and 100 μs, and an acquisition delay time (dead time) of 300 μs was used, in order to have a nonobservable effect from the ring down in the probe after 10^6 transients.

The longitudinal relaxation rate, R_1, was determined by the inversion recovery technique,[6] and the transverse relaxation rate, R_2, was obtained from the line width at half-height, $\Delta\nu_{1/2}$, by using the relation $R_2 = \pi\Delta\nu_{1/2}$.

Theoretical Section

The magnetic relaxation of a spin ≥1 nucleus is, in most cases, due to the interaction of the nuclear electric quadrupole moment with fluctuating electric field gradients at the position of the nucleus. The relaxation of a spin $I \geq 1$ nucleus is, in general, not a simple exponential decay.[7] The decay of the longitudinal ($\alpha = 1$) and transverse ($\alpha = 2$) magnetization for a $I = {}^7/_2$ nucleus is a weighted sum of four exponentials

$$M_1(t) = M_1(\infty)(1 - K\sum_{i=1}^{4} C_{1,i} \exp(-R_{1,i}t)) \quad (1)$$

$$M_2(t) = M_2(0)(\sum_{i=1}^{4} C_{2,i} \exp(-R_{2,i}t)) \quad (2)$$

where $\sum^4_{i=1} C_{\alpha,i} = 1$ and $K = 2$ for the inversion recovery experiment. It is not possible to obtain analytical solutions of the relaxation equations, but they may be solved numerically for each $\omega\tau_c$ value[8] (ω is the resonance frequency in radian/s and τ_c is the correlation time describing the reorientation of the electric field gradients at the nucleus). A single-exponential decay is approached as the relaxation rates of the different components become increasingly alike and/or as the amplitude ($C_{\alpha,i}$) of one of the components approaches unity ($\omega\tau_c \to 0$).

For a spin $^7/_2$ nucleus, like ^{43}Ca, the decay of the longitudinal magnetization may be approximated by a single exponential for correlation times such that $\omega\tau_c \leq 10$, since $C_{11} > 0.96$ for $\omega\tau_c \leq 10$.[8] The decay of the transverse magnetization is more complex, and for $\omega\tau_c$ values ≥1 multiexponential behavior is expected (Table I). The effect of the 300-μs dead time used due to the long "ring down" of our instrument at this frequency is simply to increase the relative amplitude of the dominant component (see Table I).

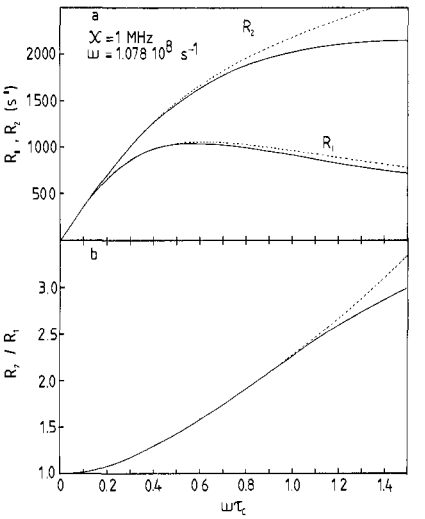

Figure 1. (a) The full lines represent the apparent relaxation rates, calculated from a fit of a simple-exponential decay (for R_1) to the numerically obtained relaxation parameters and from the half-width of the actual superposition of Lorentzian lines (for R_2). In the calculations above the effect of the finite dead time (300 μs) is taken into account. The dotted lines represent the analytical expressions for the average relaxation rates, eq 3 and 4. All data were calculated by using $\omega = 1.078 \times 10^8$ s^{-1} and $\chi = 1$ MHz. (b) The ratio R_2/R_1 as a function of $\omega\tau_c$. The data were calculated as described above.

For correlation times such that $\omega\tau_c \simeq 1.5$, the NMR signal of a spin $^7/_2$ nucleus displays a virtual Lorentzian line shape. In fact, due to the long dead time (>300 μs) of the instrument when low γ nuclei are studied, it will be difficult to observe deviations from Lorentzian line shape at even higher $\omega\tau_c$ values.

The apparent longitudinal relaxation rates R_1 in Figure 1 were calculated from the fit of a simple exponential decay to the numerically obtained relaxation parameters and the apparent transverse relaxation rates R_2 from the half-width of the actual superposition of four Lorentzian signals. The full lines in Figure 1a show the result of this procedure, using a quadrupole coupling constant χ of 1 MHz, $\omega = 1.078 \times 10^8$ s^{-1}, and Figure 1b shows the ratio R_2/R_1 as a function of $\omega\tau_c$. The apparent relaxation rates above were calculated taking the effect of the 300-μs dead time into account (Table I).

It has recently been shown,[9] by a perturbation treatment, that simple analytical expressions for the relaxation rates exist for "nearly exponential" relaxation. The result, which is identical with the average relaxation rates $\langle R_1 \rangle$ and $\langle R_2 \rangle$ derived by McLachlan,[10] is given by eq 3 and 4 for the case of quadrupolar

$$\langle R_1 \rangle = \frac{3\pi^2}{10}\chi^2\frac{2I+3}{I^2(2I-1)}\left[\frac{0.2\tau_c}{1+(\omega\tau_c)^2} + \frac{0.8\tau_c}{1+(2\omega\tau_c)^2}\right] \quad (3)$$

$$\langle R_2 \rangle = \frac{3\pi^2}{10}\chi^2\frac{2I+3}{I^2(2I-1)}\left[0.3\,\tau_c + \frac{0.5\tau_c}{1+(\omega\tau_c)^2} + \frac{0.2\tau_c}{1+(2\omega\tau_c)^2}\right] \quad (4)$$

(5) Forsēn, S.; Lindman, B. "Ion Binding in Biological Systems Measured by Nuclear Magnetic Resonance", In "Methods of Biochemical Analysis"; Glick, D., Ed.; Wiley: New York, 1981.
(6) Farrar, T. C.; Becker, E. D. "Pulse and Fourier Transform NMR"; Academic Press: New York, 1971.
(7) Lurcat, F. *C. R. Hebd. Acad. Sci.* **1955**, *240*, 2402.
(8) Bull, T. E.; Forsēn, S.; Turner, D. L. *J. Chem. Phys.* **1979**, *70*, 3106.
(9) Halle, B.; Wennerström, H. *J. Magn. Reson.* **1981**, *44*, 89.
(10) McLachlan, A. D. *Proc. R. Soc. London, Ser. A* **1964**, *280A*, 271.

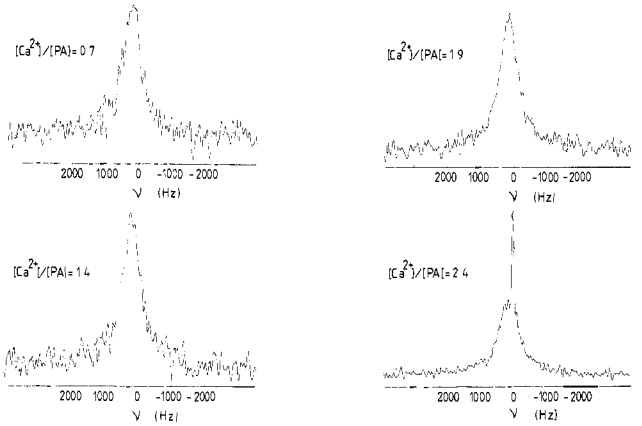

Figure 2. The ^{43}Ca NMR spectrum of a 5.6 mM PA solution, at 23 °C and pH 7.3, at different $[Ca^{2+}]/[PA]$ ratios. The spectra were recorded by using a 70-μs (90°) pulse, a data acquisition time of 20 ms and an instrument dead time of 300 μs. Each spectrum required ca. 2×10^6 transients corresponding to ~12 h of accumulation.

relaxation. Results of calculations based on these equations are given in Figure 1 as the dotted lines, and the agreement between the analytical expressions and the numerically obtained apparent relaxation rates is good up to $\omega\tau_c \sim 1.0$.

The direct observation of the ^{43}Ca NMR signal from the Ca^{2+} ions bound to proteins is possible either when the exchange conditions are slow (i.e. when the exchange rate, k_{off}, between the protein binding sites and the bulk solution is slow compared to the relaxation rate on the protein) or when the association constant is sufficiently high to ensure that all of the Ca^{2+} ions are bound to the protein for a low Ca^{2+} concentration. In practice this restricts us to direct observations of ^{43}Ca resonances from Ca^{2+} binding sites with association constants greater than $\sim 10^6$ M^{-1}.

Results

Parvalbumin. Parvalbumins form a group of relatively small muscle proteins ($M_r = 11.5 \times 10^3$) found in significant amounts in the muscles of aquatic vertebrates.[11] The crystal structure of the carp component, pI = 4.25, is known from X-ray studies. Parvalbumin contains two high affinity cation binding sites that bind Ca^{2+} and Mg^{2+} with association constants of the order of 10^9 and 10^5 M^{-1}, respectively.[12] In these sites, generally termed the EF and CD sites of PA, the Ca^{2+} ions are octahedrally coordinated by carbonyl and carboxylate oxygen atoms (in the EF site one water oxygen coordinates to the Ca^{2+} ion). In addition to the sites described above, ^{113}Cd NMR measurements shows the presence of a "third" cation binding site for carp, pI = 4.25 (PA), which binds monovalent ions ($K_w \approx 10$ M^{-1}) and divalent ions ($K_w \approx 3 \times 10^2$ M^{-1}). This site is located near one of the strong sites, in fact less than 7 Å away (Swärd et al., to be submitted for publication). At the present time it is not possible to say if this site is located near the EF or CD site, since the basis for the assignment of the ^{113}Cd signals from PA[13] is still uncertain.

Figure 2 shows the ^{43}Ca NMR signal for different $[Ca^{2+}]/[PA]$ ratios at 23 °C. The signals seen for $[Ca^{2+}]/[PA]$ ratios less than 2 are the ^{43}Ca resonances from Ca^{2+} ions bound to the EF and CD sites of PA. In these spectra no ^{43}Ca signal is observed from free Ca^{2+} ions since the concentration of these ions is exceedingly low under the experimental conditions. The ^{43}Ca signal is shifted 10 ppm downfield relative to the signal of free Ca^{2+}. The line shape appears to be Lorentzian, with a line width at half-height,

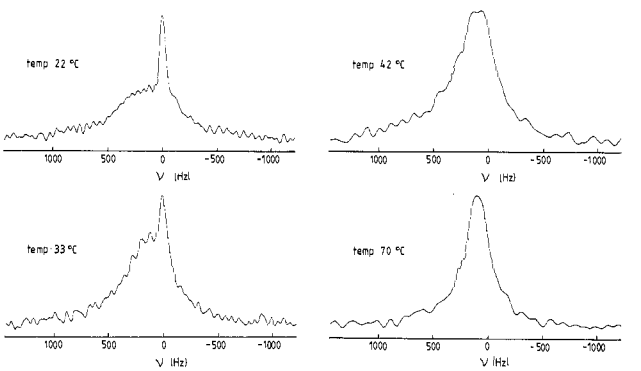

Figure 3. The temperature dependence of the ^{43}Ca NMR spectrum of a 5.6 mM PA solution, pH 7.3, at a $[Ca^{2+}]/[PA]$ ratio of 2.4. The data were recorded by using a pulse repetition rate of 40 ms, a dead time of 300 μs, and a 70-μs pulse (90°).

$\Delta\nu_{1/2}$, of 670 ± 30 Hz. At $[Ca^{2+}]/[PA]$ ratios greater than 2, an additional narrow ^{43}Ca NMR signal emerges. This signal is 64 Hz broad at room temperature (23 °C) while the ^{43}Ca signal in the absence of added protein is 10 Hz. This second ^{43}Ca signal is therefore broadened due to interaction with the EF and CD sites and/or by the "third" site.

Figure 3 shows the temperature dependence of the ^{43}Ca spectrum at a $[Ca^{2+}]/[PA]$ ratio of 2.4. At increasing temperatures the ^{43}Ca signal from "free" Ca^{2+} is broadened, while the line width of the "protein-bound" signal decreases. The decrease in the line width of the ^{43}Ca signal is partly an effect of the correlation time τ_c (cf. eq 4 and Figure 1), which most likely describes the reorientation of the entire protein molecule, and is partly due to the increasing rate of exchange with the free Ca^{2+} ions.

The broadening of the ^{43}Ca signal from the "free" Ca^{2+} ions can readily be explained by chemical exchange between the EF and/or CD sites of PA and the bulk solution. If the exchange rate, k_{off}, is slow compared to the relaxation rate, R_2, at the binding site, the observed line width of "free" Ca^{2+} increases with increasing exchange rate (i.e., temperature). A rough estimate of the exchange rate of Ca^{2+} at 23 °C can be obtained from a band-shape analysis of the spectra in Figure 3. The result of the fitting is $k_{off} = 20$ s^{-1}. The question may be raised whether the "third" site contributes significantly to the observed broadening of the "free" Ca^{2+} signal. This type of weak cation site is not likely to bind Ca^{2+} in such a way that slow exchange conditions would apply. The ratio $k_{on}/k_{off} = K_w$ gives a value of $k_{off} \geq 3 \times 10^5$ s^{-1} ($k_{on} \geq 10^8$ s^{-1} M^{-1},[14] $K_w = 3 \times 10^2$ M^{-1}) which is much greater than the expected value of the relaxation rate of ^{43}Ca at the "third" site. ^{43}Ca ions interacting with this site would then show a "fast exchange" behavior in variance with the observed behavior; cf. Figure 3.

Figure 4a shows some partially relaxed ^{43}Ca NMR spectra of the protein-bound Ca^{2+}. Figure 4b shows the intensity of the signals as a function of the delay time, τ, and the data were fitted as a single-exponential decay. The result of the fitting procedure is shown as the full line, corresponding to the longitudinal relaxation rate $R_1 = (1.6 \pm 0.1) \times 10^3$ s^{-1}. The transverse relaxation rate was estimated from the ^{43}Ca signal line width to be $R_2 = (2.1 \pm 0.1) \times 10^3$ s^{-1}. All data were measured at 23 °C. From these data, using Figure 1b, a correlation time of 4.0 ± 1 ns is obtained, a value which is in good agreement with the rotational correlation time calculated from the Debye–Stokes–Einstein equation (Table II). The ^{43}Ca correlation time and the transverse relaxation rate, inserted into the numerical solutions or into eq 4, give a value of the quadrupole coupling constant $\chi = 1.3 \pm 0.2$ MHz.

A longer rotational correlation time for PA, 12 ns, has been reported by Nelson et al.[15] from ^{13}C NMR measurements.

(11) Pechère, J.-F. In "Calcium-Binding Proteins and Calcium Function"; Wasserman, R. H., Corradion, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., Siegel, F. L., Eds.; Elsevier/North Holland: New York, 1977; p 213.

(12) Cox, J. A.; Wnuk, W.; Stein, E. A. In "Calcium Binding Proteins and Calcium Function"; Wasserman, R. H., Corradion, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., Siegel, F. L., Eds.; Elsevier/North Holland: New York, 1977; p 266.

(13) Drakenberg, T.; Lindman, B.; Cavé, A.; Parello, J. *FEBS Lett.* **1978**, *92*, 346.

(14) Eigen, M.; Hammes, G. G. *Adv. Enzymol. Relat. Subj. Biochem.* **1963**, *25*, 1.

Table II. Some Physical Constants and ^{43}Ca NMR Data for PA, CaM, and TnC

protein	$10^3 M_r$, D	hydrodynamic radius R_H, nm	τ_{rot},[a] ns	R_2/R_1	τ_c, ns	δ	χ, MHz
parvalbumin	11.5	1.5	2.9	1.35 ± 0.15	4.0 ± 1	+10 ± 1	1.3 ± 0.2
calmodulin	16.8	2.1	8.0	2.05 ± 0.35	8.2 ± 2	+10 ± 1	1.15 ± 0.05
troponin C	17.8	2.2	9.3	2.45 ± 0.45	11.0 ± 2	+10 ± 1	1.05 ± 0.05

[a] For a sphere of radius R_H, obeying the Debye-Stokes–Einstein relation $\tau_{rot} = 4\pi\eta R_H^3/(3\,KT)$, where $T = 296$ K and $\eta = 8.50 \times 10^{-4}$ kg (ms)$^{-1}$.

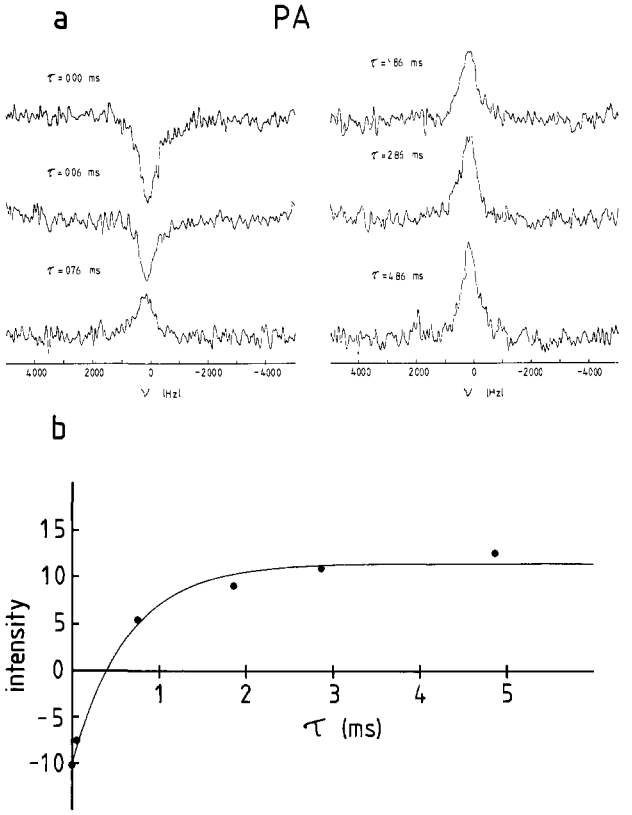

Figure 4. (a) The ^{43}Ca NMR spectrum of a 5.6 mM PA solution, 23 °C and pH 7.3, at a $[Ca^{2+}]/[PA]$ ratio of 1.9 after different delay times in the inversion recovery sequence. (b) The intensities of the signals as a function of the delay time. The solid curve represents the result of fitting the data to the equation $Y = A(1 - B\exp(-t/T_1))$, resulting in $A = 11.6$, $B = 1.9$, and $T_1 = 0.64$ ms.

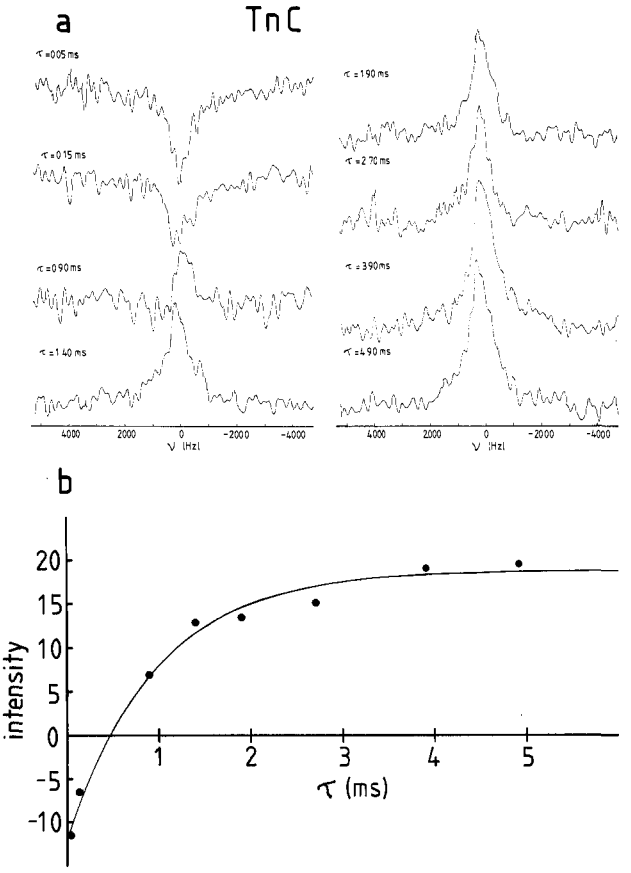

Figure 5. (a) Some partially relaxed ^{43}Ca NMR spectra of a 1.0 mM TnC solution, pH 7.2, containing 1.75 mM Ca^{2+}. The spectra were taken at 23 °C. Each spectrum was recorded by using a pulse interval of 10 ms, a 90° pulse of 50 μs, and a "ring down" time of 300 μs. For each spectrum a total of 2×10^6 transients were recorded during ca. 6 h of accumulation. (b) The intensities of the signals above as a function of the delay time τ. The solid line represents a fit of the equation given in the caption to Figure 4 to the data, yielding $A = 19.0$, $B = 1.6$, and $T_1 = 0.95$ ms.

However, their data where obtained at a higher protein concentration, 15 mM (~20 mass %), which should be compared to 5.6 mM (~8 mass %) used in our ^{43}Ca measurements. The difference in the two correlation times is most likely due to that at the higher PA concentration, protein—protein interaction has a nonnegligible effect on the rotational diffusion. This argument is supported by ^1H NMR and ^{17}O NMR measurements,[16,17] demonstrating that at protein concentrations greater than ~10 mass % interaction between the macromolecules becomes significant.

Troponin C. Troponin C (TnC), the Ca^{2+}-binding component of the troponin complex in vertebrate skeletal muscles, is a low molecular weight protein (18×10^3). TnC has been reported to possess several classes of cation-binding sites: two "high affinity" sites that bind Ca^{2+} and Mg^{2+} with association constants of 2×10^7 and 5×10^3 M^{-1}, respectively, and two weaker "regulatory" binding sites ($K_{Ca} = 3 \times 10^5$ M^{-1}).[18] These two types of sites are believed to display similarities with the EF and CD sites of PA, based on sequence analogies.[19] As for PA there is evidence for other weaker cation sites.[3,18,20] Figure 5a shows some partially relaxed ^{43}Ca NMR spectra of a TnC solution ($[Ca^{2+}]/[TnC] = 1.7$) at 23 °C. Under the conditions used only the "high affinity" sites will be populated, and the signal originates from the Ca^{2+} ions bound to these two sites. The signal is shifted 10-ppm downfield relative to the signal of free Ca^{2+} ions and exhibits a Lorentzian line shape with a line width at half-height of 750 ± 30 Hz, corresponding to $R_2 = (2.4 \pm 0.1) \times 10^3$ s^{-1}. The data in Figure 5 can be analyzed to obtain the longitudinal relaxation rate. A nonlinear fit of the intensities results in $R_1 = (1.0 \pm 0.1) \times 10^3$ s^{-1}. The resultant ratio $R_2/R_1 = 2.45 \pm 0.45$ corresponds to a correlation time τ_c of 11.0 ± 2 ns. The value of the correlation time most likely corresponds to the correlation time for the rotational diffusion of the protein molecule (Table II). Using the value of the correlation time, we can estimate a quadrupole

(15) Nelson, D. J.; Opella, S. J.; Jardetzky, O. *Biochemistry* **1976**, *15*, 5552.
(16) Oakes, J. *J. Chem. Soc., Faraday Trans. 1* **1976**, *72*, 216.
(17) Halle, B.; Andersson, T.; Forsén, S.; Lindman, B. *J. Am. Chem. Soc.* **1981**, *103*, 500.
(18) Potter, J. D.; Gergely, J. *J. Biol. Chem.* **1975**, *250*, 4628.
(19) Collins, J. H.; Potter, J. D.; Horn, M. J.; Wilshire, G.; Jackman, N. *FEBS Lett.* **1973**, *36*, 268.
(20) Hincke, M. T.; McCubbin, W. D.; Kay, M. *Can. J. Biochem.* **1978**, *56*, 384.

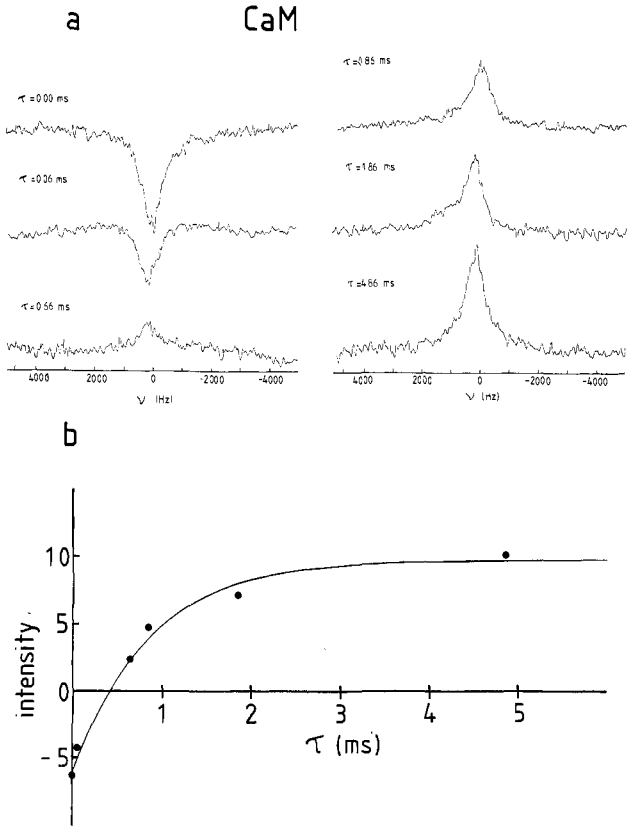

Figure 6. (a) The ^{43}Ca NMR spectrum of a 2.8 mM CaM solution, pH 7.3, containing 1.7 mol of Ca^{2+}/mol of protein, at different delay times in the inversion recovery sequence. Each spectrum was recorded by using a pulse interval of 20 ms, a pulse width of 100 μs (90°), and an instrumental dead time of 300 μs. For each spectrum of 2×10^6 transients were recorded during ca. 12 h of accumulation. (b) The intensity of the signals above as a function of the delay time. The solid line represents the result of fitting the data to the equation given in the caption to Figure 4, yielding $A = 9.8$, $B = 1.6$, and $T_1 = 0.86$ ms.

coupling constant of 1.05 ± 0.05 MHz from the numerically obtained average relaxation rate.

At higher $[Ca^{2+}]/[TnC]$ ratios the ^{43}Ca signal shifts upfield and the line width decreases because of chemical exchange effects from the other sites of TnC.[3]

Calmodulin. Calmodulin (CaM) is a low molecular weight protein ($M_r = 16.7 \times 10^3$) which acts as a universal regulator of several enzyme systems. The modulation of enzymatic activities takes place after a conformational change induced by the binding of Ca^{2+}.[21] As for TnC, CaM has been shown to possess four regions with an amino acid sequence analogous to those of the EF and CD sites of PA.[22] The values of the Ca^{2+} binding constants are still debated,[21,23] but ^{113}Cd measurements indicate the presence of two high affinity sites comparable to those of TnC.[4]

Figure 6a shows some partially relaxed ^{43}Ca NMR spectra of a CaM solution, $[Ca^{2+}]/[CaM] = 1.7$, at 23 °C. The observed ^{43}Ca NMR signal is due to the Ca^{2+} ions bound to the "high affinity" sites of CaM. Under the conditions used, only the "high affinity" sites will be populated according to published binding constants.[21,23] As for PA and TnC the resonance is shifted 10-ppm downfield relative to the signal of free Ca^{2+} and has a line width of 770 ± 50 Hz ($R_2 = (2.4 \pm 0.2) \times 10^3$ s^{-1}). Similar to the TnC case, the ^{43}Ca resonance shifts toward free Ca^{2+} as the $[Ca^{2+}]/[CaM]$ ratio is increased above 2. This effect is attributed to chemical exchange of the bulk Ca^{2+} ions with the weaker cation sites on CaM (Andersson et al., manuscript in preparation). The data in Figure 6a can be analyzed to obtain the longitudinal relaxation rate, and a nonlinear fit of the intensities results in $R_1 = (1.2 \pm 0.1) \times 10^3$ s^{-1}. Using the relaxation data and the numerically calculated average relaxation rates or eq 3 and 4, we find a correlation time of $\tau_c = 8.2 \pm 2$ ns and a quadrupolar coupling constant χ of 1.15 ± 0.05 MHz. The value of the correlation time is in good agreement with the rotational correlation time, $\tau_c = 8.0$ ns, calculated from the Debye–Stokes–Einstein equation, using a Stokes radius of 20.9 Å[21] (Table II).

Concluding Discussion

The chemical shift range of ^{43}Ca is ca. 60 ppm, ±30 ppm relative to free Ca^{2+} ions in aqueous solution.[24] The chemical shifts of $^{43}Ca^{2+}$ ions bound to the three proteins all show the same shift value and a similar magnitude of the quadrupole coupling constant (Table II). This observation supports the arguments, based on sequence homologies, that the Ca^{2+} binding sites in these proteins have the same arrangements of oxygen ligands coordinating to the Ca^{2+} ion.[25]

Measurements of R_1 and R_2 for the "protein-bound" ^{43}Ca NMR signals results in correlation times that are in good agreement with the values expected for the rotational correlation time (see Table II). This finding may be taken to indicate that internal mobility at the Ca^{2+} sites is restricted and considerably slower than the nanosecond time scale.

The use of ^{43}Ca NMR in the study of Ca^{2+}–protein interactions has been shown to be possible by using specially designed "high-field" instruments in combination with isotopically enriched $^{43}Ca^{2+}$. At reasonably high protein concentrations, observations of the "protein-bound" ^{43}Ca resonances can be made. For reasonably short correlation times ($\omega\tau_c \leq 1.5$), information about the correlation time and the quadrupole coupling constant may be obtained from numerically fitted apparent relaxation rates. The analytical equations (eq 3 and 4) for the average relaxation rates have been shown to be valid up to $\omega\tau_c \approx 1.0$. For correlation times such that $\omega\tau_c > 2$, a numerical fit of the signal line shape to the relaxation equations is necessary. In this type of calculations the effect of the instrument dead time has to be taken into account.

The currently long "ring down" time (>300 μs) of NMR spectrometers operating at this frequency (17.16 MHz) results in a loss of ~50% of the signal intensity ($\chi = 1$ MHz) due to relaxation during the dead time. An additional disadvantage is that a long dead time makes it difficult to observe a non-Lorentzian line shape. This indicates a need for probe constructions that lead to shorter "ring down" times.

Acknowledgment. We thank Bertil Halle and Häkan Wennerström for valuable discussion on the relaxation theory. T.A. gratefully acknowledges a grant from Stiftelsen Bengt Lundqvists Minne.

Registry No. Troponin C, 64540-33-6; Ca^{2+}, 14127-61-8.

(21) Klee, C. B.; Crouch, T. H.; Richman, P. G. *Annu. Rev. Biochem.* **1980**, *49*, 489.
(22) Watterson, D. M.; Sharief, F.; Vanaman, T. C. *J. Biol. Chem.* **1980**, *255*, 962.
(23) Crouch, T. H.; Klee, C. B. *Biochemistry* **1980**, *19*, 3692.
(24) Drakenberg, T. *Acta Chem. Scand., Ser. A*, in press.
(25) It has recently been pointed out that second-order effects may influence the NMR relaxation parameters and chemical shifts under nonextreme narrowing conditions (Werbelow, L. G.; Marshall, A. G. *J. Magn. Reson.* **1981**, *43*, 443). It can however be shown (H. Wennerström, personal communication) that the neglect of these effects will result in negligible errors in the relaxation parameters derived under the conditions applicable to this work: $\omega\tau_c \leq 1.5$, $\omega \simeq 10^8$ s^{-1} and $\chi = 1$ MHz. The dynamic frequency shift, however, has to be considered to contribute to the chemical shift change by 5–7 ppm for PA.