^{113}Cd NMR IN THE STUDY OF CALCIUM BINDING PROTEINS: TROPONIN C

Sture FORSÉN, Eva THULIN and Hans LILJA
Physical Chemistry 2, Chemical Centre, POB 740, S-220 07 Lund, Sweden

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1. Introduction

Calcium is one of nature's most versatile metal ions, for example some 70 calcium binding proteins have been described [1]. However there exists a marked lack of physical probes to study the properties of Ca^{2+} in these molecules in solution. Ca^{2+} is diamagnetic and has no convenient optic spectroscopic properties. The magnetic isotope ^{43}Ca(I = 5/2; natural abundance 0.145%) may in principle be studied by NMR methods but direct observation of ^{43}Ca NMR signals from calcium binding proteins is most probably ruled out by the large nuclear electric quadrupole moment of ^{43}Ca. Due to very efficient quadrupole relaxation the NMR signal is expected to be broadened beyond detectability in binding sites with less than cubic symmetry [2,3]. The indirect NMR method, i.e. the study of NMR signals from small ions in solution that undergo reasonably fast chemical exchange with macromolecular binding sites, has proved very informative for ions such as Na^+ and Cl^- [4]. However it is not expected to be generally applicable for the study of Ca^{2+} binding proteins since if the calcium binding constant exceeds 10^4 M^{-1} the chemical exchange rate of Ca^{2+} between protein and solvent is expected to be too slow at room temperature.

^{113}Cd NMR has in recent years emerged as a useful tool for the study of zinc proteins [5–9]. ^{113}Cd has a spin I = 1/2 magnetic nucleus and Cd^{2+} can often substitute for Zn^{2+} in zinc enzymes with at least partial retention of biological activity [10]. The ionic radius of Cd^{2+} (0.097 nm) is close to that of Ca^{2+} (0.099 nm) and it may be expected that Cd^{2+} could substitute also for Ca^{2+} in calcium binding proteins. This has been demonstrated [11] in a study of carp parvalbumin. Here we show how ^{113}Cd NMR can be applied to study the calcium binding muscle protein troponin C. This study has been greatly facilitated by recent instrumental developments that are further described below.

Abbreviations: TnC, rabbit skeletal muscle troponin C; NMR, nuclear magnetic resonance; CPA, carp parvalbumin; FT, Fourier transform; Tris, tris(hydroxymethyl)aminomethane

2. Materials and methods

TnC was prepared from 1 kg rabbit back and leg muscles by the procedure in [12] with the difference that after the DEAE–Sephadex separation the TnC containing fraction was filtrated through a Sephadex G-100 column (4 × 35 cm) in 0.05 M cacodylate buffer at pH 6.4. The purity was checked by agarose gel electrophoresis and the same method used to study the Ca^{2+} binding capacity. In SDS disc gel electrophoresis [13] we obtained one band corresponding to mol. wt 19 000. The absorption coefficient A_{280}/A_{260} was 0.89. Calcium-free TnC was prepared by slowly passing 10 ml of a 0.1 mM solution of TnC in 0.05 M cacodylate buffer at pH 6.4 through a Chelex-100 column (1 × 10 cm). The Ca^{2+} content of TnC after this treatment, as measured by atomic absorption spectroscopy, was < 0.1 mol Ca^{2+}/mol protein.

A 0.1 M solution of $Cd(ClO_4)_2$ was prepared by dissolving CdO (96.3% isotope enriched in ^{113}Cd, Oak Ridge Nat. Lab., USA) in 1 M $HClO_4$ and neutralid with Tris buffer to a final pH 6.4.

The ^{113}Cd FT NMR spectra were obtained at 56.55 MHz on a NMR spectrometer partly constructed

in our laboratory. The instrument is equipped with a widebore (89 mm bore diam. at room temp.) superconducting magnet (Oxford Instr., Oxford) operating at 6.0 Tesla. In order to achieve maximum sensitivity for the weak ^{113}Cd NMR signals the probe is equipped with a receiver/transmitter solenoid with its axis transverse to the magnetic field and into which the sample can be inserted from the side. The sample volumes used were mostly 2–3 ml. The non-spinning resolution of this probe is about 5 Hz which is more than adequate for our present purposes. For each NMR spectrum some 10 000–20 000 transients were obtained. Signal aquisition time was 60 ms and time between pulses was 0.5 s except in the T_1 determinations. All chemical shifts are reported relative to 0.1 M $Cd(ClO_4)_2$, shifts to low fields taken as positive. All spectra were obtained at 23°C sample temperature.

3. Results

Aliquots of a 0.1 M $Cd(ClO_4)_2$ solution (96.3% isotope enriched in ^{113}Cd) were successively added to a 2.8 mM solution of calcium-free TnC at pH ~6.4. At a molar ratio Cd^{2+}/TnC of 1:1 two ^{113}Cd NMR signals of ~30 Hz line width were observed at δ = −107.5 ppm (signal A) and −111.0 ppm (signal B). The chemical shifts were slightly pH dependent. At higher molar ratios Cd^{2+}/TnC these signals increased in intensity with no significant change (± 0.3 ppm) in chemical shift. At molar ratios exceeding ~4:1 an additional broad ($\Delta\nu_{1/2} \approx 450$ Hz) ^{113}Cd NMR signal was observed near δ = 31–33 ppm; this signal was found to move towards lower fields and become slightly more narrow at still higher Cd^{2+}/TnC ratios. The ^{113}Cd NMR spectrum at a Cd^{2+}/TnC ratio of 2:1 is shown in fig.1.

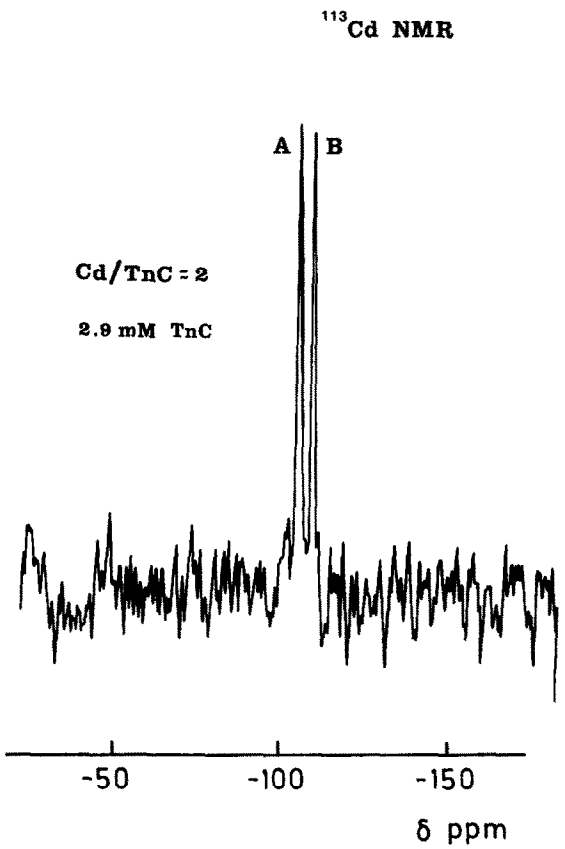

Fig.1. The ^{113}Cd FT NMR spectrum at 56.55 MHz of a solution containing 2.9 mM rabbit muscle troponin C (TnC) and 6 mM $Cd(ClO_4)_2$ (96.3% isotope enriched in ^{113}Cd) in a 0.05 M cacodylate buffer solution at pH 6.4. The spectrum was obtained after 20 000 transients, the acquisition time was 0.060 s and the time between pulses 0.5 s. A 6.0 Tesla superconducting magnet was used and the total volume of the sample, placed transversely into the magnetic field, was 2.8 ml.

Addition of $MgCl_2$ to a solution with a Cd^{2+}/TnC ratio of 6:1 (TnC 0.5 mM, pH 6.4) to ~2 M total Mg^{2+} conc. caused no significant change of the chemical shift of signals A and B but the intensity of these signals was reduced by ~50%.

The longitudinal relaxation time, T_1, of the signals A and B was determined using the saturation recovery technique [14] on a solution with a Cd^{2+}/TnC ratio of 3:1 (TnC 1.9 mM, pH 6.2). A least squares fit gave the results $T_1^A = 1.6 \pm 0.2$ s and $T_1^B = 1.8 \pm 0.2$ s. These measurements, which were extended to a waiting time of 10 s between the 90° pulses, took in all 3 days to perform.

The effect of additions of Ca^{2+} on the ^{113}Cd NMR spectrum was studied on a solution with a Cd^{2+}/TnC ratio of 4:1 (TnC 1.8 mM, pH 6.4). The A and B signals were observed to decrease and the broad signal at about 32 ± 1 ppm to increase in intensity as shown in fig.2a,b. The effect of additions of Gd^{3+} was studied on a 1.1 mM TnC solution (pH 5.9) with a molar ratio Cd^{2+}/TnC of 5:1. The results are summarized in fig.3.

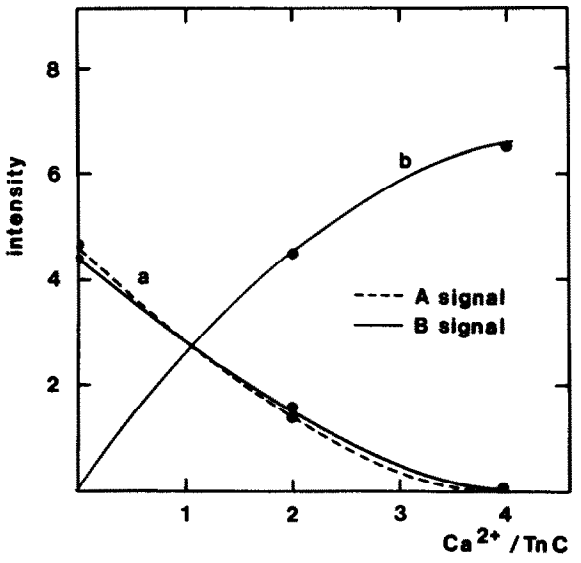

Fig.2. The dependence on the molar ratio Ca^{2+}/TnC of the intensity of (a) the ^{113}Cd NMR signals A and B (cf. fig.1) and (b) the ^{113}Cd NMR signal at about +32 ppm, attributed to 'free' Cd^{2+}. A solution of 1.8 mM TnC and 7.2 mM $Cd(ClO_4)_2$ in 0.05 M cacodylate buffer (pH 6.4) was used and aliquots of a 2.0 M solution of $Ca(ClO_4)_2$ was successively added. The intensities are relative values.

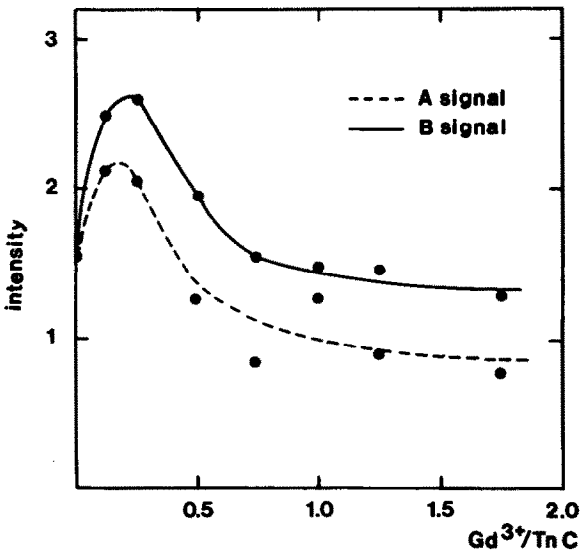

Fig.3. The dependence of the intensity of the ^{113}Cd NMR signals A and B (cf. fig.1) on the molar ratio Gd^{3+}/TnC. A solution of 1.1 mM TnC and 5.5 mM $Cd(ClO_4)_2$ in 0.05 M cacodylate buffer (pH 5.9) was used and aliquots of a 80 mM $GdCl_3$ was successively added. At the arrow precipitates were formed. The intensities are relative values.

4. Discussion

Rabbit skeletal muscle troponin C (TnC) has been reported to possess two classes of Ca^{2+} binding sites [15]; two sites with a high affinity for Ca^{2+} (K_{Ca} = 2.1×10^7 M^{-1}) but that also bind Mg^{2+} (K_{Mg} = 3.2×10^5 M^{-1}) and two sites that bind Ca^{2+} specifically but with lower affinity ($K_{Ca} = 3.2 \times 10^5$ M^{-1}). We tentatively attribute the two ^{113}Cd NMR signals A and B (cf. fig.1) at $\delta_A = -107.5$ ppm and $\delta_B = -111.0$ ppm to Cd^{2+} bound to the two non-equivalent high affinity sites (the Ca^{2+}/Mg^{2+} sites). The chemical shifts of the A and B signals are characteristic of Cd^{2+} coordinated to oxygen atoms [16] and similar to those attributed to the 'CD' and 'EF' sites in $(Cd)_2$-CPA [11]. This result fits nicely with the similarity in the Ca^{2+} binding oxygen ligands observed for CPA [17] and those tentatively suggested for the 2 Ca^{2+}/Mg^{2+} sites in TnC on the basis of the primary structure [18–20]. Four carboxylate ligands (Asp or Glu) should thus be involved in both proteins but the 2 additional ligands of the coordination octahedra differ both between the 2 proteins and the 2 individual sites on each protein.

From the results of the addition of Mg^{2+}, and provided the decrease in ^{113}Cd signal intensity really reflects a decreased binding and not an increased T_1 (cf. below), it can be estimated that the ratio $K_{Cd^{2+}}/K_{Mg^{2+}}$ must be of the order of 5×10^2. The competition experiments with Ca^{2+} (fig.2) indicate the affinity of TnC for Cd^{2+} to be lower than for Ca^{2+} in linewidth predictions [21] for calcium binding proteins with oxygen ligands. The results of the Mg^{2+} and Ca^{2+} competition experiments taken together do not seem to be compatible with the ratio $K_{Ca^{2+}}/K_{Mg^{2+}} \approx 10^2$ reported for the Ca^{2+}/Mg^{2+} binding site of TnC [15]. Our present data are somewhat preliminary, however.

The intensity of the ^{113}Cd NMR signals A and B as a function of added Cd^{2+} indicate the binding sites to have very similar affinity for Cd^{2+}. Measurements of relative intensities in FT NMR are however only reliable when the longitudinal relaxation times T_1 of individual signals are equal, or when the time between the r.f. pulses is much longer than the longest T_1 encountered. The latter approach is impractical in the present case but the T_1 measurements show the A and B NMR signals to have the same T_1 within the

experimental error and the intensity measurements in the just discussed experiment should be reasonably reliable. The near identity of K^{A}_{Cd} and K^{B}_{Cd} may be an indication of a positive cooperativity between the corresponding sites.

In CPA, Ca^{2+} in the EF site has been shown to be selectively replaceable by Tb^{3+} and a similar behaviour has been inferred for $(Cd)_2$-CPA for which the ^{113}Cd signal assigned to the EF site can be selectively reduced by the addition of Gd^{3+} [11]. This difference in affinity for lanthanides of the two CPA sites can presumably be linked to the occurrence of a H_2O molecule as a ligand in the EF site. In the tentatively assigned ligands of the Ca^{2+}/Mg^{2+} sites of TnC no H_2O molecule is involved [20]. (A H_2O ligand may however be involved in one of the Ca^{2+}-specific sites of TnC [20]). The result of fig.3 is strikingly different from that observed for $(Cd)_2$-CPA. The initial increase in ^{113}Cd NMR signal intensity may be due to a Gd^{3+}-mediated conformational change that increases the affinity of the Ca^{2+}/Mg^{2+} sites for Cd^{2+} (it should be remembered that under the conditions of our experiment these sites are not 100% occupied by Cd^{2+} and that some 'free' Cd^{2+} is always present). An alternative interpretation is that the paramagnetic Gd^{3+} binds, specifically or unspecifically, sufficiently close to the Cd^{2+} binding sites to reduce the ^{113}Cd longitudinal relaxation time. Under the conditions of our experiments (cf. section 2) such a reduction would result in increased signal amplitudes. This point will be further investigated.

The near independence of the linewidth of the ^{113}Cd NMR signals A and B of the ratio Cd^{2+}/TnC indicates the chemical exchange rate of Cd^{2+} between 'free' and bound states to be slow on the NMR time scale (off-rates $< 10^2$ sec^{-1}) in agreement with findings for $(Cd)_2$-CPA. Our failure to observe ^{113}Cd signals from the 2 additional Ca^{2+} binding sites of TnC (the Ca^{2+}-specific sites, if our present tentative assignment of the A and B signals is correct) may be due to faster chemical exchange in this case broadening these signals beyond detectability. The finding that the signal from 'free' Cd^{2+} observed near $\delta = +32$ ppm narrows at Cd^{2+}/TnC ratios $> 4:1$ is in line with this hypothesis.

In conclusion it would appear that ^{113}Cd NMR can provide new information about the Ca^{2+} binding properties of TnC. It is however also evident that great caution must be exercised when arguments are based on observed changes in ^{113}Cd signal intensities; it is important to distinguish between effects originating in altered T_1:*s* and those due to population changes.

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