A ^{113}Cd NMR STUDY OF CALMODULIN AND ITS INTERACTION WITH CALCIUM, MAGNESIUM AND TRIFLUOPERAZINE

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1. Introduction

Calcium has been long recognized as an important regulator of a variety of cellular events [1–5]. The detailed mechanism whereby calcium acts is still largely unknown. However, it has been demonstrated that calcium regulation of a number of enzyme systems is mediated by a low molecular weight, thermostable protein termed calmodulin. This protein was first described [4–7] as an activator of brain cyclic nucleotide phosphodiesterase. Calmodulin has subsequently been found in tissues from various organs in both vertebrate, invertebrate and plant species [3,8,9,9a]. There is good evidence that calmodulin represents an ubiquitous calcium regulatory protein whose primary sequence is highly conserved throughout all eucaryotic cells [3].

Calmodulin (M_r 16 700) consists of a single polypeptide chain whose amino acid composition is characterized by having a large number of acidic residues (glutamic and aspartic), a lack of cysteine and tryptophan, and the presence of one mole of the unusual amino acid trimethyllysine [10]. Calmodulin's sequence is homologous with that of parvalbumin and skeletal troponin C (TnC) and like the latter its amino acid sequence can be divided into 4 internally homologous domains, each of which has a potential calcium binding site [10]. The calcium binding properties of calmodulin have been subject to several studies and while there is some inconsistency as regards the relative number of high and low affinity sites most studies indicate that calmodulin will bind 4 mol calcium/mol protein [11–15]. Experimental evidence from a number of studies indicate that calcium binding to calmodulin is accompanied by pronounced changes in its solution conformation [11–14, 16–22]. The biological activity of calmodulin is inhibited by certain antipsychotic drugs of the phenothiazine type, the most effective being trifluoperazine which has been reported to bind to calmodulin with a binding constant of $\sim 10^6$ M^{-1} [23].

Here we have utilized ^{113}Cd NMR to study calmodulin and its interaction with calcium, magnesium, and trifluoperazine. ^{113}Cd has a spin $I = 1/2$ magnetic nucleus amenable for study by NMR and the similarity of the ionic radii of Cd^{2+} (0.097 nm) and Ca^{2+} (0.099 nm) makes Cd^{2+} a fairly good substitute for calcium in calcium binding proteins. The applicability of ^{113}Cd NMR in the study of calcium binding proteins has been demonstrated for carp parvalbumin [24] and skeletal muscle TnC [25].

2. Materials and methods

Two sources of calmodulin were used: bovine brain and testes. Calmodulin was prepared from bovine brain essentially as in [15] with the exception that the second and third steps were omitted. Bovine testes calmodulin was prepared by a modification of the procedure in [13] which did not include the heating step. Briefly this method included ammonium sulfate precipitation, chromatography on DEAE-cellulose at pH 7.5 followed by chromatography on Sephadex G-75 superfine (K. S., in preparation). The purity of the calmodulin was checked by SDS gel electrophoresis and by its ability to activate Ca^{2+}, Mg^{2+}-ATPase from erythrocytes as determined using the assay in [31]. The ^{113}Cd NMR results obtained on calmodulin from bovine brain and testes are very similar, however we cannot rule out the possibility that the small differences observed are due to artifacts arising from the

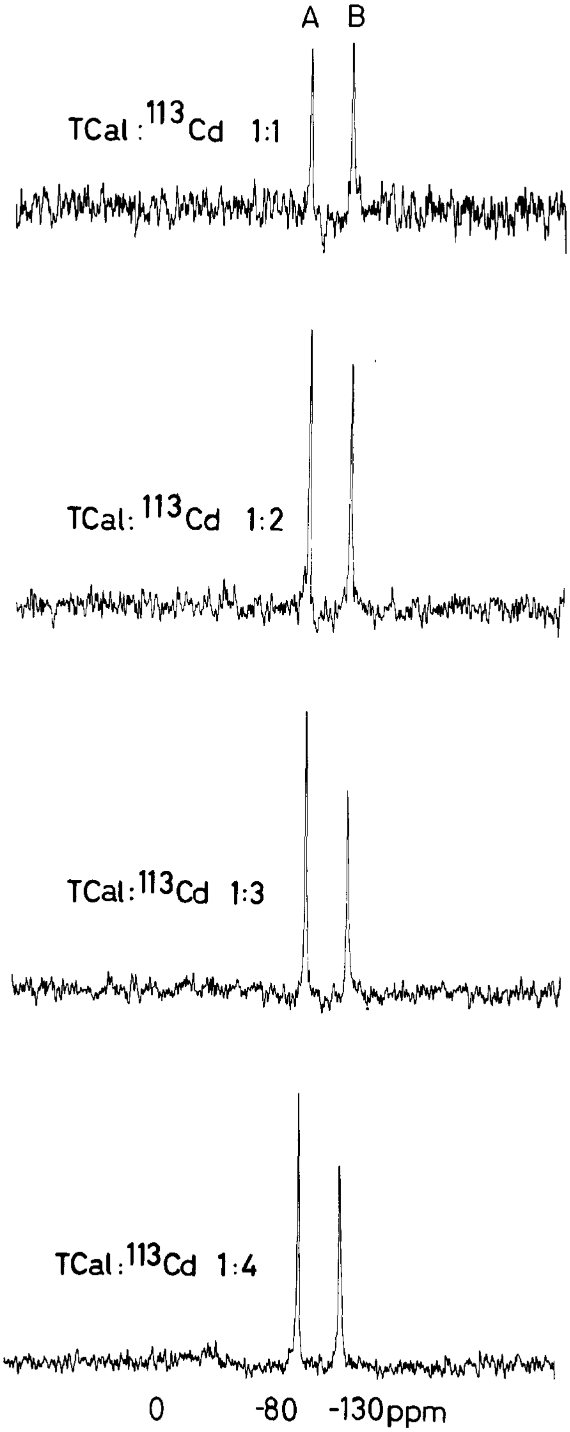

Fig.1. The ^{113}Cd NMR spectra at 56.55 MHz of a solution containing 1.52 mM of calcium free bovine testes calmodulin (TCal) to which successive amounts of $^{113}Cd^{2+}$ is added. No signals from 'free' $^{113}Cd^{2+}$ ($\delta \approx 0$ ppm) are observed at $^{113}Cd^{2+}$/TCal ratios <4:1.

two different preparation procedures rather than to minor differences in the primary sequence of the protein.

Calcium-free calmodulin was prepared by passing an aqueous protein solution through a column of Chelex-100 (10 ml/50 mg protein). The solution was then lyophilized and dissolved in 10 mM Tris–perchlorate at pH 7.0. The calcium content of calmodulin after this treatment, as measured by atomic absorption spectroscopy, was ~0.2 mol calcium/mol protein.

Trifluoperazine was obtained from Smith, Kline, and French Labs., Philadelphia, PA and was used without further purification. The ^{113}Cd NMR spectra were obtained at 56.55 MHz as in [25]. All chemical shifts are reported relative to 0.1 M $Cd(ClO_4)_2$, shifts to low field being taken as positive.

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 1 mM solution of calcium free bovine brain calmodulin (BCal) at pH 7.0. Two ^{113}Cd NMR signals at −88.5 ppm (signal A) and −115.0 ppm (signal B) were observed at a molar ratio of Cd^{2+}/BCal of 1:1. Signals A and B increased in intensity, in parallel, with increasing Cd^{2+}/BCal ratios with no significant chemical shift change and reached their maximum intensity at a Cd^{2+}/BCal ratio of ~2:1. The line width of the signals was ~50 Hz. At higher Cd^{2+}/BCal ratios no change in the chemical shifts of signals A or B was observed, however, the signals broadened and a broad signal at ~−5 ppm appeared. At Cd^{2+}/BCal ratios >6:1 a precipitate was observed in the sample tube.

The above experiment was repeated with bovine testes calmodulin (TCal) (1.5 mM, pH 7.0). For Cd^{2+}/TCal ratios ⩽2:1 the results were virtually identical with those obtained with the bovine brain calmodulin (fig.1). At Cd^{2+}/TCal ratios from 2:1–4:1 no broadening of the A and B signals was observed; nor was there any evidence of a broad ^{113}Cd NMR signal appearing in the chemical shift region around $\delta = 0$.

The longitudinal relaxation time, T_1, of signals A and B was determined using the progressive saturation technique on a solution with a Cd^{2+}/BCal ratio of 4:1 (BCal 1.0 mM, pH 7.0). A least-squares fit to the data gave the results; $T_1^A = 0.26 \pm 0.05$ s and $T_1^B = 0.28 \pm 0.05$ s.

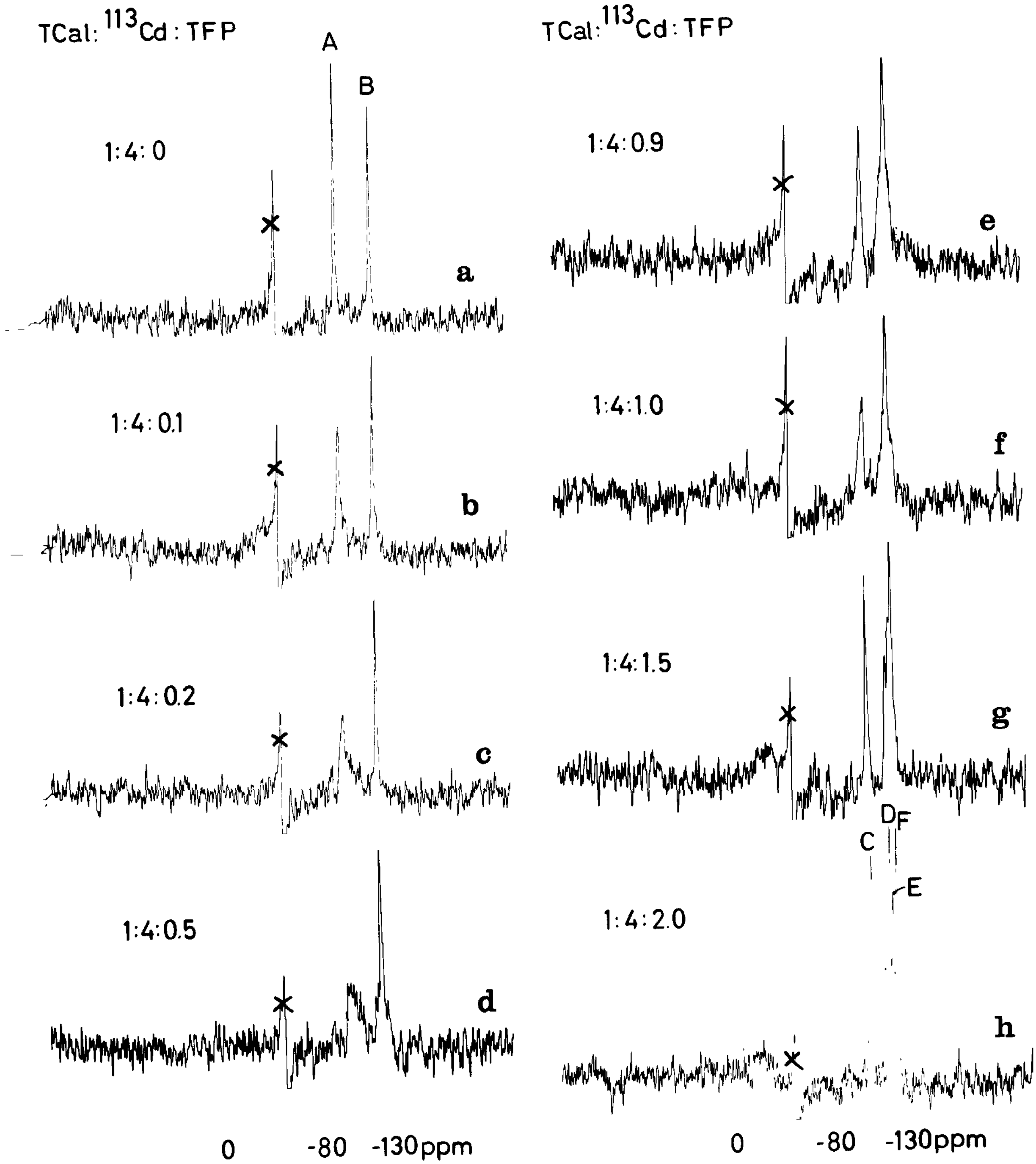

Fig.2. The effects of trifluoperazine (TFP) on the ^{113}Cd NMR spectrum of a 1.39 mM solution of $(Cd)_4$-calmodulin (bovine testes). Note that effects can be observed already at 0.1 mol TFP/mol TCal. At a TFP:$(Cd)_4$-TCal ratio of 2:1, 4 narrow ^{113}Cd NMR signals appear at −99.7 ppm (C), −113.5 ppm (D), −115.8 ppm (E) and −118.7 ppm (F). At still higher ratios the E and F signals tend to overlap (cf. fig.3, top). The peak marked with a cross is an instrumental artefact due to an improperly balanced quadrature detector.

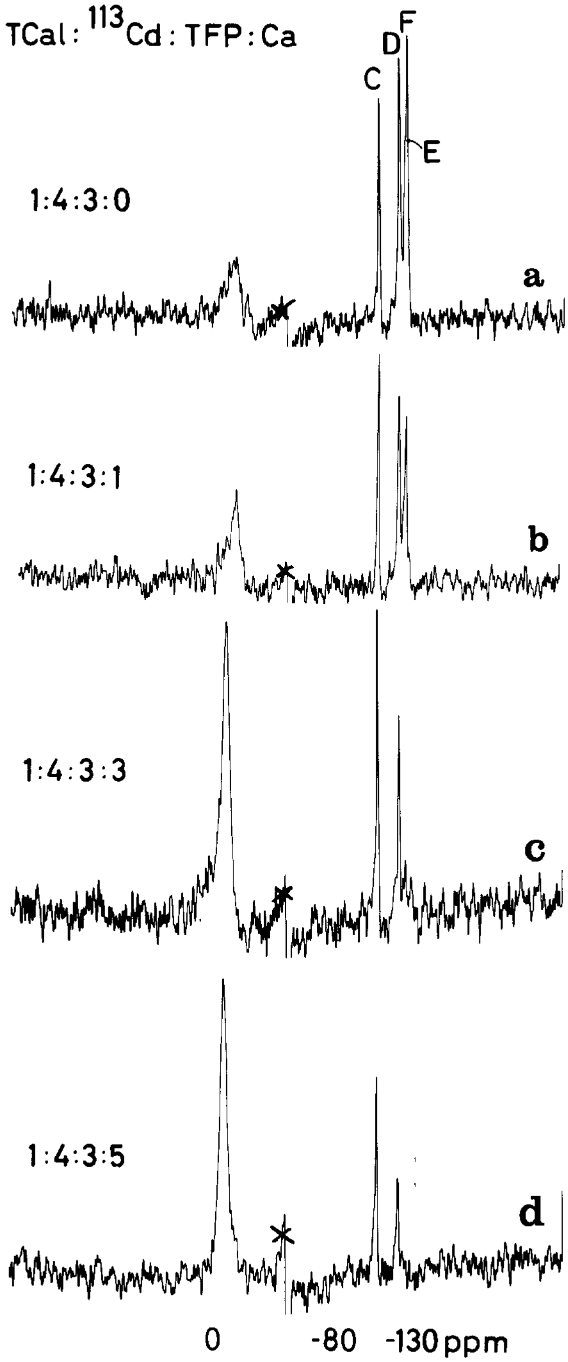

Fig.3. The effects of successive additions of Ca^{2+} on the ^{113}Cd NMR spectrum of a solution containing 1.46 mM $(Cd)_4$-TCal and 4.38 mM TFP. The reduction of the intensity of the ^{113}Cd NMR signals from the bound $^{113}Cd^{2+}$ is accompanied by the appearance of a broad signal at $\delta \approx 0$ ppm attributed to 'free' $^{113}Cd^{2+}$. As in fig.2 a spurious peak due to an improperly balanced quadrature detector is observed in some spectra.

The successive addition of Ca^{2+} to a sample with the Cd^{2+}/TCal ratio of 4:1 resulted in a decrease of both ^{113}Cd NMR signals A and B, the latter being more affected than the first. At a Ca^{2+}/TCal ratio of 2:1 the B signal had almost disappeared in the noise whereas signal A retained ~50% of its initial intensity. Signal A could still be observed after the addition of 5 equiv. Ca^{2+} with an intensity of ~25% of the initial intensity.

Addition of $MgCl_2$ to a solution with a Cd^{2+}/BCal ratio of 4:1 (BCal 1 mM, pH 7.0) to total $[Mg^{2+}]$ ~50 mM caused no significant change in the chemical shifts or the intensities of the A and B ^{113}Cd NMR signals.

A 0.05 M solution of trifluoperazine (TFP) was successively added to samples with either brain or testes calmodulin (Cal ≈ 1 mM, pH 7.0) with Cd^{2+}/Cal ratios of 4:1. The accompanying change in the ^{113}Cd NMR spectrum for the testes calmodulin solution is shown in fig.2. The brain calmodulin sample showed very similar effects, the broadening of the ^{113}Cd signals were, however, larger and for some TFP concentrations the signals disappeared in the noise. The chemical shifts of the ^{113}Cd signals in the presence of 3 equiv. TFP were identical for brain and testes calmodulin.

To solutions with the TFP/Cd^{2+}/Cal ratio 3:4:1 (i.e., the final solutions in the above TFP titration) small amounts of $CaCl_2$ were successively added. The different ^{113}Cd NMR signals were dissimilarly affected, as shown in fig.3 for testes calmodulin.

4. Discussion

The ^{113}Cd NMR spectra observed when $^{113}Cd^{2+}$ is successively added to calcium-free bovine brain and testes calmodulin indicate the presence of two high affinity Cd^{2+} sites somewhat differing in properties (signals A and B of fig.1). The chemical shifts of signals A and B fall in the region expected for cadmium bound solely to oxygen ligands and are similar but not identical, with the shifts observed for $(Cd)_2$-parvalbumin [24] and $(Cd)_2$-troponin C [25]. These findings are in line with the conjectures made concerning the general nature of Ca^{2+} binding sites calcium-modulated proteins [29,30].

The $^{113}Cd^{2+}$ titration results do not rule out the existence of Cd^{2+} binding sites in addition to the high affinity ones. In the case of bovine testes calmodulin the results are particularly clear-cut. Since no signal from 'free' cadmium ($\delta \approx 0$) could be detected below a Cd^{2+}/TCal ratio of 4:1 there appears to exist a total of 4 cadmium binding sites. ^{113}Cd NMR signals from the third and fourth site are not observable presumably due to substantial chemical exchange broadening. These results obtained with bovine testes calmodulin are in many respects similar to those obtained with TnC [25] for which binding studies indicate the presence of 2 sites with high and 2 with medium affinity for Ca^{2+}.

Since the intensities of the ^{113}Cd NMR signal A and B are observed to increase in parallel as $^{113}Cd^{2+}$ is successively added to calcium free calmodulin, this indicates that the individual high affinity binding constants, K_{Cd}^{A} and K_{Cd}^{B}, are almost identical or that the 2 high affinity sites display positive cooperativity [26]. The same type of behaviour is observed for the 2 high affinity sites of rabbit skeletal muscle TnC [25].

The high affinity Cd^{2+} binding sites of calmodulin have a very high affinity for Ca^{2+} as shown by the dependence of the intensity of the ^{113}Cd NMR signals A and B on the molar ratio Ca^{2+}/Cal. Results obtained with bovine testes calmodulin indicate that the relative affinities K_{Ca}/K_{Cd} are somewhat larger for the high affinity site corresponding to the B signal than for the site corresponding to the A signal.

Mg^{2+} is much less efficient than Ca^{2+} in displacing $^{113}Cd^{2+}$ from the high affinity sites of calmodulin. A rough estimate indicates the Mg^{2+} binding constant of these sites to be at least 2 orders of magnitude smaller than the Ca^{2+} binding constants. The calcium and magnesium binding studies [11,27] also indicate similar differences in the affinity of Ca^{2+} and Mg^{2+}.

The addition of the phenothiazine drug TFP to $(Cd)_4$-calmodulin causes quite dramatic changes in the ^{113}Cd NMR spectrum (fig.2). A broadening of the signals is observed at a TFP/Cal ratio of 0.1:1; with a maximal broadening at TFP/Cal ratios close to 0.5:1. At still higher ratios there emerges an entirely new ^{113}Cd NMR spectrum with 4 substantially narrower signals, two of which eventually coalesce (cf. fig.3). The spectral changes in the ^{113}Cd NMR spectrum of fig.2a through 2h may be rationalized by assuming that TFP is exchanging between the calmodulin molecules with a rate such that the average lifetime of a calmodulin–TFP complex is $\sim 10^{-3}$ s. Computer simulations assuming different sets of connectivities between the initial sites/signals and final sites/signals *C–F*, produce theoretical line shapes very similar to those experimentally observed. It appears that the binding of TFP to calmodulin causes the average conformation of the latter to change in such a way that all 4 Cd^{2+} binding sites are affected. The reduced linewidth of the ^{113}Cd signal at high TFP/Cal ratios is most likely a result of a reduced chemical exchange rate between free and bound Cd^{2+}. These results are in agreement with the equilibrium dialysis results in [23] where the addition of Ca^{2+} to the calcium free calmodulin caused the TFP binding constant to increase ~10-fold. A corollary to this result is that addition of TFP to calmodulin should cause a corresponding increase in the Ca^{2+} binding constant. Phenothiazins and hydrophobic fluorescent dyes bound competitively to calmodulin in a calcium-dependent manner in [32]. This result further suggests that this binding site may represent a site of interaction between calmodulin and its associated binding proteins.

It has also been shown that troponin-I binding to troponin C [33] and calmodulin [32] results in an increased affinity of the protein for Ca^{2+}. This is similar to these results for TFP binding to calmodulin; that is a decreased off-rate for Cd^{2+} which would imply an increased binding constant. It is tempting to speculate that the binding of either calmodulin-activated proteins or phenothiazines to calmodulin will result in similar increases in the affinity of calmodulin for Ca^{2+} through a common mechanism. Calmodulin also bound TFP molecules [23]. Our results do not contradict this but do indicate that <2 TFP molecules/calmodulin is sufficient to cause most, if not all, of the conformational changes affecting the 4 divalent cation binding sites.

The conformational changes in calmodulin resulting from binding of TFP indicated here provides a basis for studying inhibitory effects of TFP on a variety of calmodulin-activated biochemical processes. The Ca^{2+} affinity of the sites corresponding to signals *C–F* is clearly different as shown by the Ca^{2+} titration results in the presence of TFP. A structural interpretation of this finding must await an assignment of the individual ^{113}Cd NMR signals to the cation binding regions of calmodulin.

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