Characterization of the calcium-binding sites of calcineurin B

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Abstract Calcineurin (CaN) is a calcium- and calmodulin-dependent serine/threonine phosphatase whose inhibition by the immunosuppressant-immunophilin complexes (cyclosporin-cyclophilin and FK506-FKBP) is considered key to the mechanism of immunosuppression. CaN is a heterodimer, consisting of a 59 kDa catalytic subunit (A) and a 19 kDa calcium-binding regulatory subunit (B). The latter is postulated to harbor four calcium binding domains of the EF hand type. The titration of the CaN B apoprotein with the isomorphic Cd^{2+} was followed by ^{113}Cd NMR and these data support one high-affinity metal binding site and three lower-affinity ones. Flow dialysis data with Ca^{2+} indicate one high affinity calcium binding site with $K_d \sim 2.4 \times 10^{-8}$ M and three other sites with $K_d \sim 1.5 \times 10^{-5}$ M. The chemical shifts of all four ^{113}Cd resonances (−75, −93, −106 and −119 ppm) are in the same range as found in other ^{113}Cd substituted calcium-binding proteins, and are indicative of all-oxygen coordination of pentagonal bipyramidal geometry.

Key words: Calcineurin; Calcium binding; EF hand; Cadmium-113 NMR

1. Introduction

Calcineurin (CaN), also known as protein phosphatase 2B, is a Ca^{2+}- and calmodulin (CaM)-dependent serine/threonine phosphatase consisting of a 59 kDa catalytic subunit (A) and a 19 kDa regulatory subunit (B) that binds Ca^{2+} [1]. CaN is inhibited by the immunosuppressant–immunophilin complexes of cyclosporin (CsA)-cyclophilin (CyP) and FK506-FKBP [2]. Apparently, CaN inhibition blocks the translocation and assembly of the nuclear transcription factor of activated T cells (NF-AT), thus preventing the transcription of cytokine genes and T cell activation [3]. CaNB is essential for the CaN phosphatase activity [1], and for the binding of immunosuppressant-immunophilin complexes to CaN [4]. The regulation of the CaN phosphatase activity by intracellular Ca^{2+} is mediated by the calcium-binding proteins CaNB and CaM which interact with CaNA at distinct sites in a tight trimeric complex [1]. On the basis of its amino acid sequence, CaNB is postulated to harbor four calcium-binding sites of the EF hand type [5], similar to the helix–loop–helix motif of other calcium-binding proteins such as troponin C (TnC) and CaM [6]. Metal-binding sites of Cd^{2+}-substituted metalloproteins can be probed by ^{113}Cd NMR, thus taking advantage of the favorable characteristics of this spin-1/2 nucleus [7–9]. This metal ion substitution is especially homologous for calcium-binding proteins since the 0.99 Å-radius Ca^{2+} is isomorphically replaced by the 0.97 Å-radius Cd^{2+} ion. We have thus undertaken a ^{113}Cd NMR investigation of CaNB, in conjunction with calcium flow dialysis experiments to quantitate the metal binding affinities and associate them with the specific metal binding sites.

2. Materials and methods

2.1. Preparation and purification of recombinant CaNB (rCaNB)

Myristoylated rCaNB was expressed and isolated from the *E. coli* strain BL21(DE3) [10] containing the plasmids pBB131 [11] and pRCNBT775–3 [12]. The plasmid pBB131, kindly provided by Dr. Jeffrey Gordon, Washington University, St. Louis, MO, contains the gene for *Saccharomyces cerevisiae* myristoyl-coenzyme A:protein *N*-myristoyltransferase (E.C. 2.3.1.97) under control of the *tac* promoter while pRCNB775–3 contains the gene for rat calcineurin B (Genbank accession number L03554) under control of the T7 promoter. The protein was purified from cells grown in 15 liter of LB broth using anion exchange and gel filtration chromatographies as described [12]. The protein appeared homogeneous as judged from Coomassie blue-stained 13% sodium dodecylsulfate polyacrylamide gels.

2.2. NMR sample preparation and protein-Cd titration

Since purified rCaNB has a tendency to readily oligomerize via thiol oxidation and crosslinking, the two free cysteine residues of the recombinant protein were carboxymethylated. The protein was first concentrated to 6.5 mg/ml using an Amicon stirred cell equipped with a Diaflo YM3 membrane (Amicon, Beverly, MA) followed by the addition of two equivalents of β-mercaptoethanol (final concentration, 670 μM). After a 30 min incubation at 30°C, iodoacetic acid was added to 65 mM, the pH of the sample raised to approximately 8 with solid Tris, and the sample incubated for an additional 30 min at room temperature. The reaction was then quenched by incubating the sample for 30 min at room temperature after adding β-mercaptoethanol to 130 mM. Carboxymethylated CaNB was fully functional toward potentiating the phosphatase activity of CaNA (data not shown). The protein was first dialyzed overnight against 1.0 liter of 25 mM HEPES, pH 7.0 buffer with Chelex-100 resin (Bio-Rad, Hercules, CA), and then overnight against water at 4°C. It was then concentrated under N_2 by ultrafiltration to ~2 ml using an Amicon Microconcentrator equipped with a YM-10 membrane at 4°C and buffer-exchanged against 5 ml of 0.02% NaN_3 in 90% H_2O/10% D_2O, pH 6.2 (henceforth referred to as the solvent). The apo-rCaNB was reconstituted by successive additions of aliquots from a $^{113}CdCl_2$ (96.3 atom% ^{113}Cd) stock solution prepared by dissolving ^{113}CdO (Oakridge National Laboratory) in conc. HCl, evaporating the solvent, and taking up the salt in water. In order to avoid possible interactions of Cd^{2+} with buffer components that could affect ^{113}Cd chemical shifts and linewidths, no buffer was used. During the titration, the requisite quantity of $^{113}CdCl_2$ in 5 ml solvent was

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Abbreviations: CaN, calcineurin; CaNA, calcineurin A; CaNB, calcineurin B; rCaNB, recombinant CaNB; CaM, calmodulin; CsA, cyclosporin A; CyP, cyclophilin; FKBP, FK506 binding protein; NFAT, nuclear factor of activated T cells; Tris, tris(hydroxymethyl)-aminomethane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TnC, troponin C; DTT, dithiothreitol; NMR, nuclear magnetic resonance.

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gradually added to the protein via the exchange compartment of the Amicon Microconcentrator. The final NMR samples consisted of ca. 2.0 to 2.2 ml of 0.9–1.0 mM rCaNB. Protein concentrations were determined with the Bio-Rad protein assay using BSA as a standard [13]. The Cd content of the $^{113}CdCl_2$ stock solution, the protein samples and the Amicon filtrates was measured by atomic absorption, using a Perkin Elmer 3030 spectrophotometer.

2.3. NMR measurements

Proton (500.13 MHz) and cadmium-113 (110.92 MHz) NMR spectra were acquired on a Bruker AM500 NMR spectrometer at 5, 25 or 35°C, using a 5 mm proton-selective or a 10 mm broadband probe, respectively. For the proton-undecoupled ^{113}Cd NMR spectra, approximately 34K transients were recorded with 8K complex points over a 20 kHz spectral width, using a 55° nutation angle and a 2 s interpulse delay. Spectra were processed on a Silicon Graphics Indigo workstation using the FELIX software package (Biosym Technologies, San Diego, CA). The ^{113}Cd NMR spectra were referenced to external 0.1 M $Cd(ClO_4)_2$ in 90% H_2O/10% D_2O (0.00 ppm).

2.4. Flow dialysis experiments

Myristoylated rCaNB for flow dialysis experiments was made Ca^{2+}-free by exhaustive dialysis against flow dialysis buffer (20 mM HEPES, 0.2 M KCl, 1 mM DTT, pH 7.5) with Chelex-100 resin (Bio-Rad, Hercules, CA) outside the dialysis bag. Glassware and plasticware used in the Ca^{2+}-binding experiments were acid-washed prior to use in order to minimize contaminating metals. Distilled water for washing and buffer preparation was tested for calcium prior to use and found to be calcium free by atomic absorption spectrophotometry. Dialysis tubing was boiled in 10 mM EDTA followed by extensive washing with distilled water. A portion of the dialyzed apoprotein, tested for the presence of calcium using atomic absorption spectrophotometry, had < 0.03 mol Ca^{2+} bound/mol CaNB.

Flow dialysis was carried out as described [14] using a modified flow dialysis unit purchased from Spectrum Technologies. Briefly, the upper buffer chamber contained 5 μM Ca^{2+}-free CaNB and 5 μM $^{45}CaCl_2$ in a total volume of 2.2 ml Ca^{2+}-free flow dialysis buffer. The upper and lower buffer chambers were separated by a 12–14 kDa cut-off dialysis membrane. The flow rate of flow dialysis buffer through the lower chamber was 6 ml/min, with the effluent diverted to a fraction collector.

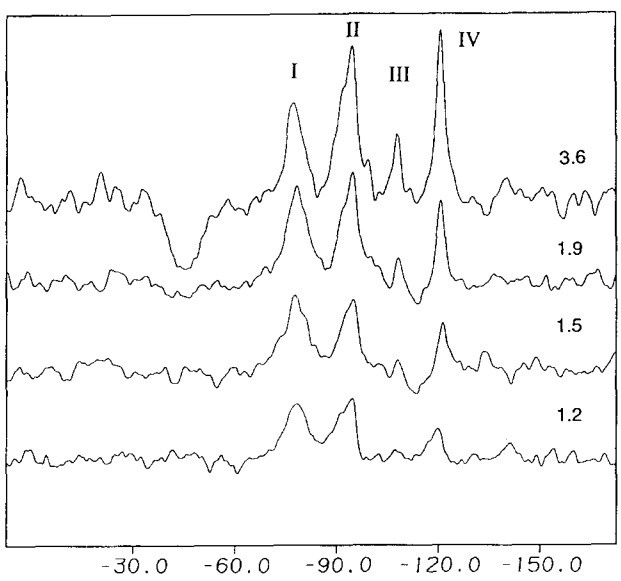

Fig. 1. Cadmium-113 NMR spectra (110.9 MHz) of 1 mM recombinant rat CaNB titrated with $^{113}CdCl_2$ at the indicated metal-to-protein molar ratios. The spectra were recorded in unbuffered aqueous solutions at pH 6.3, 25°C and referenced vs. external 0.1 M $Cd(ClO_4)_2$. The inverted downfield resonance of spectrum D is attributed to the free-bound, exchange-averaged ^{113}Cd signal that cannot be properly phased because of aliasing.

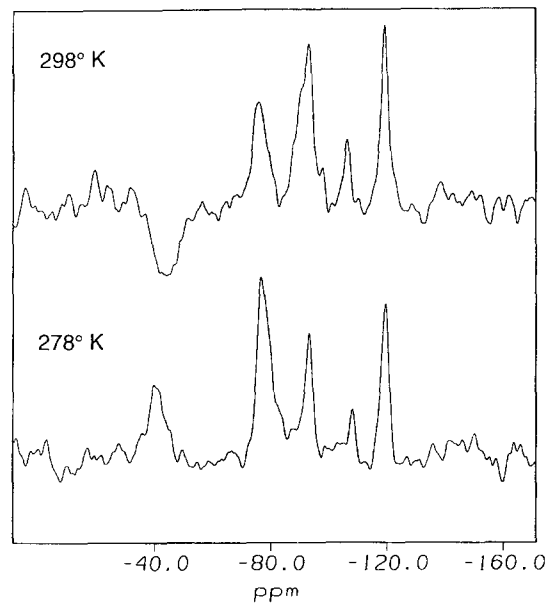

Fig. 2. Temperature dependence of the ^{113}Cd NMR of 1 mM CaNB in the presence of 3.6 equivalents of $^{113}Cd^{2+}$.

The entire experiment lasted approximately 1.5 h. Only 5–8% of the $^{45}Ca^{2+}$ initially present in the upper chamber was depleted in the experiment, an amount not expected to affect binding parameters appreciably [27].

In order to obtain an estimate of the dissociation constants for Ca^{2+} binding to CaNB and the number of each binding site, data from flow dialysis experiments were fit by a least squares procedure to the equation [15–17]:

$$\bar{\nu} = \sum_i n_i K_i[Ca^{2+}]/(1 + K_i[Ca^{2+}]) \quad (1)$$

where $\bar{\nu}$ is the number of moles of Ca^{2+} per mol of CaNB, n_i refers to the number of binding sites with binding constant K_i, and $[Ca^{2+}]$ refers to the free Ca^{2+} concentration.

3. Results

The successive addition of $^{113}CdCl_2$ aliquots to apo-CaNB gave rise to four ^{113}Cd NMR resonances at −75, −93, −106, and −119 ppm (resonances I–IV, respectively, Fig. 1). Further addition of metal, resulted in the eventual replacement of these resonances by a single broad, exchange-averaged, downfield ^{113}Cd resonance at ca. +60 ppm. Lowering the temperature from 25°C to 5°C, at the level of 3.6 equivalents of added Cd^{2+}, caused a measurable narrowing of the two most downfield resonances, presumably because of the slowing of dynamic processes that modulate these $^{113}Cd^{2+}$ signals (Fig. 2). The intensity/area variation of the ^{113}Cd resonances as a function of added $^{113}Cd^{2+}$ (Fig. 3), indicates that the most downfield resonance (−75 ppm) corresponds to a strong binding site that is saturated at a 2:1 metal-to-protein molar ratio whereas the other three resonances arise from weaker metal binding sites that are only partially occupied at the metal-to-protein ratios examined.

The binding of Ca^{2+} to CnB measured by flow dialysis showed a biphasic nature indicative of high and lower affinity sites (Fig. 4). For $[Ca^{2+}] \leq 3$ μM, a single binding site is evident while for higher $[Ca^{2+}]$, three additional sites become popu-

lated. A least squares fit of the Ca^{2+} binding data using a model for non-interacting sites gave one binding site (n = 1.2) with K_d = 0.024 μM and three sites (n = 2.5) with K_d = 15 μM (solid curve, Fig. 4). Data at $[Ca^{2+}_{free}]$ high enough to demonstrate saturation could not be obtained since the difference between $[Ca^{2+}_{free}]$ and $[Ca^{2+}_{total}]$ was comparable to the error associated with measuring their values, a practical limitation of the flow dialysis method. A Hill plot for $[Ca^{2+}] > 10$ μM gave a Hill coefficient of 1.4, indicating that only slight cooperativity exists between the three lower affinity sites.

4. Discussion

The well-documented sensitivity of ^{113}Cd NMR chemical shifts to the identity and number of the metal ligands [7–9] allows the clear resolution of all four CaNB metal binding sites by ^{113}Cd NMR. The observed chemical shifts, being in the same range as those of other calcium-binding proteins, are suggestive of all-oxygen metal ligation of similar pentagonal bipyramidal geometry [7–9]. Generally, Cd^{2+} is an excellent structural probe for the EF hand calcium binding sites [18]; the reported exceptions of intestinal calcium-binding protein [19] and calbindin 9K [20] concern atypical EF hands. Similarly to CaM and TnC, two other calcium-binding proteins that are structurally related to CaNB, the relative intensity increase of the ^{113}Cd NMR resonances during a titration experiment [21,22] should reflect the relative affinities of the protein's metal binding sites [15,23].

On visual inspection, the ^{113}Cd NMR titration data (Fig. 1) seems to indicate three strong metal binding sites that correspond to resonances I, II and IV which develop immediately, and a weaker binding site (resonance III). This analysis does not, however, take into account possible differences in the ^{113}Cd NMR resonance relaxation times [24]. More thorough quantification of the ^{113}Cd NMR titration data (Fig. 3) suggests that the metal binding site corresponding to resonance I is stronger than the other three. Unfortunately, exchange averaging of ^{113}Cd resonances probihits extending the titration much beyond 3.6 equivalents of Cd^{2+}.

Inspection of the rat CaNB sequence reveals the 12-residue sequences that comprise the Ca^{2+} binding loops of the four

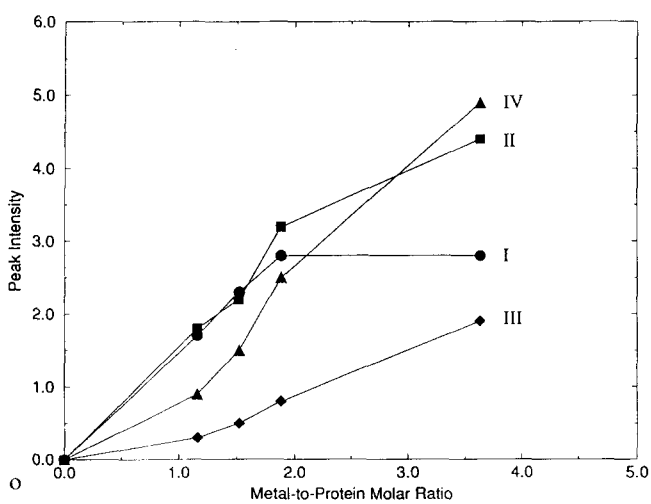

Fig. 3. Peak intensity/area increase in ^{113}Cd signals at −75 ppm (●), −93 ppm (■), −106ppm (♦), and −119 ppm (▲) upon titration of 1 mM CaNB with $^{113}CdCl_2$.

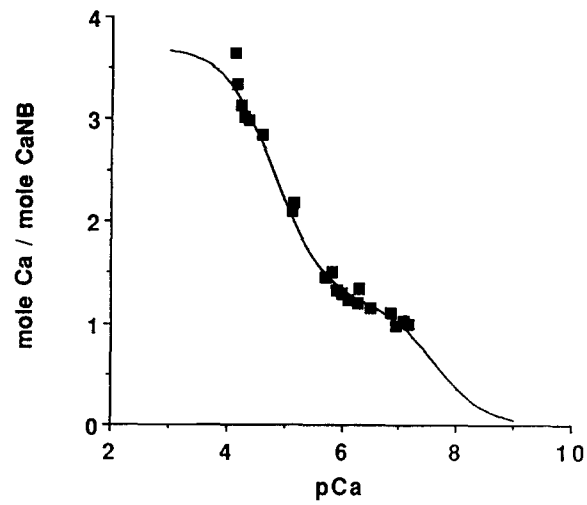

Fig. 4. A plot of mol Ca^{2+} bound/mol CaNB as a function of free Ca^{2+} concentration. The squares represent data points from two separate flow dialysis experiments. The line represents a least-squares fit to the data using Eq. 1, as detailed in the results section.

helix-loop-helix EF-hands (Table 1) which, being highly homologous to those of other calcium-binding proteins, should bind calcium via seven oxygen atoms in a pentagonal bipyramidal geometry [25]. However, the observed large variation in the calcium affinity (K_d = 10^{-5}–10^{-8}M) of the CaNB binding sites may not simply reflect the individual binding loop amino acid sequences but also those of the adjacent helices and, given that EF-hand pairs rather than single EF-hands are the functional units of calcium-binding proteins, the other EF-hand of the pair as well [26].

The presence of a single strong and three weak Ca^{2+} binding sites of isolated CaNB (this study) is in contrast with the two strong (K_d < 70 nM) and two weak Ca^{2+} binding sites of proteolyzed CaN [27], perhaps suggesting that the CaNA-CaNB interaction can also influence the affinity of metal binding sites on CaNB. In fact, using isolated unmyristoylated CaNB, Burroughs et al. measured by luminescence spectroscopy four dissociation constants for Eu^{3+} binding with values of 20 nM, 140 nM, 1.0 μM and 1.6 μM [28]. Flow dialysis of a preparation of unmyristoylated calcineurin B yielded a Ca^{2+}-binding curve identical to the one obtained here (data not shown), indicating that myristoylation does not affect metal affinity, a result found for another EF hand protein, recoverin [29]. Although part of the difference in K_d values between Eu^{3+} versus Ca^{2+} binding to CaNB can be attributed to the different valencies of the associated metal, other conditions such as ionic strength, pH and $[Mg^{2+}]$ which differ in these two studies, can affect binding interactions in the EF-hand family with metal–protein affinities varying by more than an order of magnitude under different buffer conditions [30].

Cooperative metal binding is common among Ca^{2+}-binding proteins such as CaM and TnC, enabling them to promptly regulate their biological function in response to moderate changes in Ca^{2+} concentration. Although ligand binding data with isolated CaNB revealed two types of non-interacting sites with high and lower Ca^{2+} affinities, slight (Hill coefficient = 1.4) cooperativity exists among the three low affinity sites. These data are in accord with a Hill coefficient of 1.8 for $[Ca^{2+}]$

Table 1
Sequences of the calcium-binding loops in CaNB[a]

Residues	Positions											
	1	2	3	4	5	6	7	8	9	10	11	12
31–42	D	L	D	N	S	G	S	L	S	V	E	E
63–74	D	T	D	G	N	G	E	V	D	F	K	E
100–111	D	M	D	K	D	G	Y	I	S	N	G	E
141–152	D	K	D	G	D	G	R	I	S	F	E	E

[a] Each metal should be coordinated [25] by a D1 carboxyl oxygen (+X), a D3 carboxyl oxygen (+Y), a residue 5 side chain oxygen (+Z), a water oxygen (−X), the residue 7 backbone carbonyl oxygen (−Y) and both E12 carboxyl oxygens (−Z1, −Z2).

> 10 μM observed for the Ca^{2+}-dependent activation of a proteolyzed, calmodulin-independent form of calcineurin [27]. The appearance of four ^{113}Cd resonances corresponding to the four CaNB sites during $^{113}Cd^{2+}$ titrations is not surprising since both [CaNB] and [Cd^{2+}] were millimolar or greater. With the K_d values measured by flow dialysis, all four metal sites should be appreciably occupied at [Cd^{2+}]/[CaNB] ratios of 1.0 or greater.

Both CaM and TnC are dumbbell-shaped molcules that consist of two similar globular domains, each harboring a pair of EF-hands, which are connected by a flexible helix [31,32]. It is most likely that the ongoing structure elucidation of CaNB [33] will reveal a similar protein fold. A comparison between the Ca^{2+}-filled C-domain of TnC and its Ca^{2+}-free N-domain showed a prominent hydrophobic surface that is accessible in the former but not the latter, thus implicating it in the Ca^{2+}-induced binding of target molecules [25]. Both apo-TnC and apo-CaM undergo conformational changes upon Cd^{2+} binding that are very similar to those caused by Ca^{2+} and which are readily observed by ^1H NMR [34,35]. This is also the case for the apo-CaNB studied (data not shown) although the spectral changes are obscured by the large linewidths of the protein that tends to aggregate in solution [36].

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