Structural basis for sequential displacement of Ca^{2+} by Yb^{3+} in a protozoan EF-hand calcium binding protein

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Abstract

We have studied the displacement of Ca^{2+} by the trivalent lanthanide ions (Yb^{3+}) in a protozoan (*Entamoeba histolytica*) Ca^{2+} -binding protein (*EhCaBP*), by NMR and thermodynamics. We have demonstrated, for the first time, how one can use in a combined fashion the utility of NMR and thermodynamics to have an insight to the relative binding specificities/affinity between Ca^{2+} and Yb^{3+} . As revealed by the titration experiments, Yb^{3+} displaces Ca^{2+} from the four metal binding sites present in *EhCaBP* in a sequential manner. The study provides a structural origin for such a sequential Ca^{2+} displacement by Yb^{3+} in *EhCaBP*.

Keywords: EF-hand protein; calmodulin; *EhCaBP*; pseudocontact shifts; chemical shifts; ytterbium

Supplemental material: See www.proteinscience.org.

EF-hand proteins belong to a growing family of calcium (Ca^{2+}) binding proteins. More than 1000 distinct primary sequences in this class of proteins are known and cataloged into 66 different subfamilies (Kawasaki et al. 1998). These proteins have been a subject of great interest for structural biologists, resulting in the availability of more than 100 three-dimensional (3D) structures, as of today (Nelson and Chazin 1998). They bind cooperatively to Ca^{2+} in the sub-nanomolar to millimolar range and function as signal transducers or modulators (Lewit-Bentley and Réty 2000). A few EF-hand proteins have also been proposed to be involved in extracellular functions such as cell migration, differentiation, and association (Hohenester et al. 1997). The canonical Ca^{2+} -binding motif (hereafter referred to as the Ca^{2+} -binding loop) in these proteins consists of a contiguous 12-residue loop flanked by two helices, forming the so-

called “EF-hand motif” (Kretsinger and Nockolds 1973; Strynadka and James 1989). Within the Ca^{2+} -binding loop, residues that are involved in coordination with Ca^{2+} are 1, 3, 5, 7, 9, and 12, forming a pentagonal bi-pyramidal geometry (Strynadka and James 1989), as shown below (highly conserved residues are shown and $\pm\text{X}$, $\pm\text{Y}$, $\pm\text{Z}$ refer to the vertices of the pentagonal bi-pyramid):

1	2	3	4	5	6	7	8	9	10	11	12
D	•	(D/N)	•	(D/N)	•	•	•	•	•	•	E
X		Y		Z		-Y		-X			-Z

Out of these, residues at 1, 3, 5, and 12 position coordinate directly to Ca^{2+} via their side-chain carboxylate groups, with position 1 and 12 being an invariant Asp and Glu, respectively (Strynadka and James 1989; Falke et al. 1994). Residue at position 7 coordinate to Ca^{2+} via its backbone carbonyl group, whereas residue at position 9 coordinates to Ca^{2+} indirectly through an intervening water molecule (Strynadka and James 1989).

In recent years, there has been a growing interest with regard to the factors that govern Ca^{2+} -binding affinities in EF-hand proteins (Linse and Forsén 1995). This is primarily due to the fact that a wide range of Ca^{2+} binding affinities/

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Abbreviations and symbols: NMR, nuclear magnetic resonance; ITC, isothermal calorimetry; HSQC, heteronuclear single quantum coherence; *EhCaBP*, *Entamoeba histolytica* calcium binding protein; CaM, calmodulin; TnC, Troponin C.

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specificities has been found among these proteins despite the strong conservation in their primary sequence of Ca^{2+} -binding loops (Linse and Forsén 1995). Several studies (Moeschler et al. 1980; Imaizumi et al. 1990; Linse and Forsén 1995; Gopal et al. 1997; Gilli et al. 1998) have concentrated on understanding Ca^{2+} binding affinity/specificity in these proteins against Mg^{2+} , which is the only other competitor for Ca^{2+} inside the living cell. Models based on the primary sequence of the Ca^{2+} -binding loop have been proposed to explain the origin of differences in Ca^{2+} binding affinities/specificities (Mardsen et al. 1988; Waltersson et al. 1993; Wu and Reid 1997). For example, in one of the studies, the presence of neighboring Asp residues at either the +X and +Y, the +Y and +Z, or the +X, +Y and +Z metal ion coordinating positions have been shown to lower metal binding affinity due to dentate–dentate repulsion (Mardsen et al. 1988). In another study, the side chain of the ligating residue at position +Y in the Ca^{2+} -binding loop has been shown to be important (Waltersson et al. 1993). In yet another study, EF-helices that flank the Ca^{2+} -binding loops have also been proposed to modulate the Ca^{2+} -binding affinity in this class of proteins (Sharma et al. 1997).

In the majority of these studies, the target protein/peptide has been taken in the Ca^{2+} -free (apo) form. Very few studies have focussed on the displacement of Ca^{2+} in a Ca^{2+} -saturated (holo) protein by other metal ions. Such studies yield information on the relative specificity and strength with which Ca^{2+} is bound to the protein. Tri-positive lanthanide ions (Ln^{3+}) serve as ideal candidates for the displacement of Ca^{2+} in a protein owing to their similar ionic radii as Ca^{2+} (Shannon 1976). Such a study yields valuable information on the relative binding affinities of Ca^{2+} and Ln^{3+} with the protein under investigation. Lanthanides such as Tb^{3+} and Eu^{3+} possess optical properties and facilitate structural studies in substituted proteins using fluorescence and UV-Vis absorption spectroscopy (Horrocks and Sudnick 1981; Wu et al. 1996). Another favorable property of paramagnetic Ln^{3+} is their large anisotropic magnetic susceptibility, which has been harnessed in NMR studies of Ln^{3+} -substituted proteins (Bertini et al. 2001a). The large pseudocontact shifts, observed for residues as far as 40 Å from the metal center, have been used for 3D structure refinement from NMR data (Allegrozzi et al. 2000). On the other hand, residual dipolar couplings that arise due to the partial alignment of proteins containing Ln^{3+} in high magnetic field have been used as further refinement tool (Bertini et al. 2001b) and to assess domain alignment in multidomain EF-hand proteins such as calmodulin, or CaM (Biekotsky et al. 1999). However, there have been very few studies that have aimed at understanding the binding affinity/specificity of Ln^{3+} to these proteins. To characterize the factors that govern binding of Ln^{3+} to EF-hand proteins and harness their favorable magnetic properties for structural studies by NMR, we have initiated Ln^{3+} -substitution

studies in an EF-hand Ca^{2+} -binding protein from *Entamoeba histolytica* (hereafter referred to as *EhCaBP*). A high-resolution 3D structure of this protein in solution has been recently deposited in the Protein Data Bank (Atreya et al. 2001).

EhCaBP is a 15-kD (134 amino acid residues) monomeric protein containing four canonical EF-hand Ca^{2+} -binding loops. The overall structural topology of *EhCaBP* resembles that of CaM and Troponin C (TnC) with two globular domains (the N- and C-terminal) connected by a flexible eight amino acid linker (Sahu et al. 1999a; Atreya et al. 2001). In this article, we present the NMR and thermodynamics study of Ca^{2+} displacement by Yb^{3+} in *EhCaBP*. Yb^{3+} has been chosen as the paramagnetic probe owing to its large pseudocontact shift to Curie line-broadening ratio (Bertini et al. 2001d). We have used isothermal titration calorimetry (ITC) to obtain binding affinities and free energy changes upon substitution of Ca^{2+} by Yb^{3+} . To the best of our knowledge, this is the first study involving a combined use of NMR and thermodynamics to characterize lanthanide binding in EF-hand proteins.

Results

NMR experiments

The titration of uniformly ^{15}N -labeled *EhCaBP* (hereafter referred to as [u- ^{15}N] sample) and Alanine, Leucine, and Lysine selectively ^{15}N -labeled *EhCaBP* (hereafter referred to as [ALK]- ^{15}N sample) was carried out with Yb^{3+} and the changes in 2D [^{15}N - ^1H] heteronuclear single quantum correlation (HSQC) spectrum were monitored. During the course of titration, a number of changes were noticed in the HSQC spectrum. Several new signals appeared all over the HSQC spectrum. Several of the original peaks were broadened due to the enhancement of relaxation rates induced by the paramagnetic ions (Yb^{3+}), while several others remained unaffected in their line widths during the initial steps of titration. It should be noted that when Yb^{3+} is introduced in a protein, the cross peak of any specific residue undergoes a pseudocontact shift if it lies in a distance range of ~10–25 Å of Yb^{3+} , while it is expected to completely broaden out if it is located within 10 Å of Yb^{3+} (Allegrozzi et al. 2000).

The amide ($^1\text{H}^{\text{N}}$) protons of Gly residues present at the sixth position of the four internally highly homologous Ca^{2+} binding polypeptide stretches in *EhCaBP* (Scheme 1), exhibit a characteristic downfield shift in the Ca^{2+} -bound state (holo) of the protein (Akerfeldt et al. 1996). This is the result of their involvement in hydrogen bonding with the side-chain carboxyl oxygen atom ($\text{C}'\text{O}$) of an invariant Asp at the first position of respective Ca^{2+} binding loops (shown as bold letters in Scheme 1 with numbering shown on top).

1 6 12
 MAEALFKEIDVNGDGA¹VSYEEVKAFVSKKRAIKNEQ
 LLQLIFKSIDADGNGEIDQNEFAKFYGSIQGQDLSDDKI
 GLKVLYKLMDVDGDGKLTKEEVTSFFKKHGIE
 KVAEQVMKADANGDGYITL⁶EEFLEFSL

Scheme 1

In *EhCaBP*, the corresponding Gly residues (G15, G51, G90, and G122; shown with an underline in Scheme 1) have their $^1\text{H}^\text{N}$ chemical shifts at 10.37, 10.75, 10.51, and 9.92 ppm, respectively. Thus, the (^{15}N - ^1H) cross peaks arising from these Gly residues show up in the least crowded region of the HSQC spectrum ($\omega_1 = 108.00$ – 115.00 ppm and $\omega_2 = 9.50$ – 11.00 ppm), and hence, formed the best markers for each Ca^{2+} -binding site during the course of titration. For the convenience of discussion, the four Ca^{2+} -binding sites present in *EhCaBP* are identified as follows: (a) Site I (residues 1–32), (b) Site II (residues 33–62), (c) Site III (residues 71–103), and (d) Site IV (residues 104–134).

Up to a Yb^{3+} :*EhCaBP* ratio of 1.0

During the initial stages of titration, $[\text{Yb}^{3+}]/[\text{EhCaBP}]$ ratio of (hereafter referred to as the metal:protein ratio) of 0.0–

1.0, the most important observation is the gradual disappearance of cross peaks belonging to Sites III and IV of *EhCaBP* and the gradual appearance of new pseudocontact shifted cross peaks, whereas cross peaks arising from residues at Sites I and II, are almost unaffected. As an illustrative example of this observation, Figures 1A and 1B, and 2 show selected regions of the 2D [^{15}N - ^1H] HSQC spectrum. Assignment of pseudocontact shifted peaks for residues in Site IV was obtained based on the combined use of the 2D [^{15}N - ^1H] HSQC spectra recorded on both [^{15}N] and [AKL]- ^{15}N *EhCaBP* samples as shown in Figure 3. As evident from Figure 3A, selective labeling of Ala, Leu, and Lys resulted in a dramatic simplification of the spectrum, which aided in unambiguous resonance assignments of the pseudocontact shifted peaks of residues belonging to Site IV. As an illustrative example, Figure 3B shows the assignment of pseudocontact shifted cross peaks of I107 and L126. No pseudocontact shifted peaks were observed for any of the residues belonging to Site III. To have an insight of these observations more vividly, Figure 4 shows the normalized volumes of cross peaks plotted for a few amino acid residues (both original and pseudocontact shifted peaks) in all the four metal binding sites of *EhCaBP*. These plots indicate the percentage of various species present up to a met-

Figure 1. (A–F) Selected region of 2D [^{15}N - ^1H] HSQC spectrum of *EhCaBP* showing G15, G51, G90, T98, and G122 peaks at different metal:protein ratios. The metal:protein ratio is indicated at top left in (A–F). The arrow mark in (B) and (C) points to the appearance of pseudocontact shifted peaks for G122 and G15, respectively.

al:protein ratio of 1.0. These observations taken together reveal that Yb^{3+} ions bind first to the C-terminal domain and particularly to the Site III of *EhCaBP*, resulting in complete broadening of cross peaks for residues belonging to this site. Simultaneously, pseudocontact shifts are observed for residues in Site IV, which lie within 10–25 Å of Site III. Thus, as the titration approaches a metal:protein ratio of 1.0, the third binding site is almost completely filled by the Yb^{3+} ions and a majority of $(\text{Ca}^{2+})_4\text{-EhCaBP}$ transforms into $(\text{Ca}^{2+})(\text{I})\text{-(Ca}^{2+})(\text{II})\text{-(Yb}^{3+})(\text{III})\text{-(Ca}^{2+})(\text{IV})\text{-EhCaBP}$.

From metal:protein ratio of 1.0 to 3.0

At a metal:protein ratio of 1.0, with the Site III filled almost completely with Yb^{3+} , the new Yb^{3+} added to the protein are now left with sites I, II, and IV to bind. During the second stage of titration (metal:protein ratio of 1.0–3.0), two important observations are made. They are (1) gradual and simultaneous disappearance of cross peaks belonging to sites I and II of *EhCaBP*, and (2) appearance of new pseudocontact shifted peaks for residues belonging only to Site I. As an illustrative example of this observation, Figure 1C and 1D show selected regions of the 2D [^{15}N - ^1H] HSQC spectrum. As seen in this figure, the cross peak belonging to G51 disappears almost completely as the titration approaches a metal:protein ratio of 3.0. On the other hand, a new cross peak appears at $\omega_1 = 114.6$ ppm, $\omega_2 = 8.90$

ppm, which has been assigned as the pseudocontact shifted peak of G15. This observation is substantiated further from the observation of pseudocontact shifted cross peaks of several other residues belonging to the Site I. For example, see plots of cross peak volumes for residues G13 and G51 in Figure 5 as a function of metal:protein ratio (1.0 to 4.0), which represent the intensity profiles for residues belonging to sites I and II of *EhCaBP*. These plots reveal that up to a metal:protein ratio of 3.0, there is a gradual decline in the volume of cross peaks belonging to residues in both sites I and II, while the new pseudocontact shifted cross peaks for residues in Site I gradually increase in their volumes up to a metal:protein ratio of about 2.0. Beyond this point there is gradual fall in their volumes as well. At a metal:protein ratio of 3.0 the species corresponding to Ca^{2+} -filled sites I and II has decreased to less than 5%. During this stage of titration, no pseudocontact shifts were observed for residues in Site II. Hence, this rules out the possibility of Ca^{2+} displacement from Site I by Yb^{3+} prior to that from Site II. Such observations taken together reveal that up to a metal:protein ratio of 2, Yb^{3+} binds mostly to the N-terminal domain and preferentially to Ca^{2+} -binding Site II. This results in the appearance of pseudocontact shifted peaks for residues in Site I, which lie within 10–25 Å of Site II. Further, as discussed in detail below, beyond a metal:protein ratio of 2.0, Yb^{3+} starts filling Site I of the above species, resulting in the decline in the integral volumes of pseudocontact shifted cross peaks of residues in Site I (Fig. 5).

Figure 2. Illustrative examples of pseudocontact shifted cross peaks for I107, A118, G120, G122, and L126 belonging to the Site IV of *EhCaBP* at a metal:protein ratio of 0.4.

partial Ca^{2+} displacement by Yb^{3+} in the Site IV. Thus, the titration study reveals the presence of a minor population of species $(\text{Ca}^{2+})(\text{I})-(\text{Yb}^{3+})(\text{II})-(\text{Yb}^{3+})(\text{III})-(\text{Yb}^{3+})(\text{IV})\text{-EhCaBP}$,

Figure 4. Plot of normalized volumes of original as well as pseudocontact shifted (PCS) cross peaks for three amino acid residues each in (A) Site I, (B) Site II, (C) Site III, and (D) Site IV of *EhCaBP* as a function of the metal:protein ratio.

with the major species still being $(\text{Yb}^{3+})\text{(I)}-(\text{Yb}^{3+})\text{(II)}-(\text{Yb}^{3+})\text{(III)}-(\text{Ca}^{2+})\text{(IV)}-\text{EhCaBP}$ at a metal:protein ratio of 3.0.

Beyond a metal:protein ratio of 3.0

During the final stages of titration, with both the major $(\text{Yb}^{3+})\text{(I)}-(\text{Yb}^{3+})\text{(II)}-(\text{Yb}^{3+})\text{(III)}-(\text{Ca}^{2+})\text{(IV)}-\text{EhCaBP}$ and minor $(\text{Ca}^{2+})\text{(I)}-(\text{Yb}^{3+})\text{(II)}-(\text{Yb}^{3+})\text{(III)}-(\text{Yb}^{3+})\text{(IV)}-\text{EhCaBP}$ species present at metal:protein ratio of 3.0, cross peaks belonging to Site IV gradually decrease in their integral

volumes. As an illustrative example, Figure 1E shows selected region of the 2D ^{15}N - ^1H HSQC spectrum. Further, the pseudocontact shifted cross peaks belonging to Site IV gradually decrease to ~30%, as the titration proceeds up to a metal:protein ratio of 4.0 (Fig. 6).

ITC measurements

A typical calorimetric reaction of the addition of aliquots of Yb^{3+} ligand to *EhCaBP* is shown in Figure 7A along with its ΔQ versus the metal:protein ratio in Figure 7B. Analysis of

Figure 5. Plot of normalized volumes of (A) the original and pseudocontact shifted (PCS) cross peaks for G15, and (B) the original cross peak of G51 as a function of the metal:protein ratio. These plots represent, respectively, the intensity profiles of cross peak volumes for residues in Sites I and II. The integral volumes of pseudocontact shifted cross peak of G15 plotted in (A) have been normalized with respect to the volume of its original cross peak at a metal:protein ratio of 0.

the data using the ligand-binding model by Wiseman et al. (1989), carried out using ORIGIN software, was made assuming the presence of four binding sites for Yb^{3+} in the protein. The fitting reveals three sites of endothermic in nature, while one site exhibits an exothermic behavior for the Ca^{2+} displacement by Yb^{3+} . By varying the initialization parameters used to begin the fitting procedure, it was determined that the fit was stable and no other parameter set could be found that provided a good fit to the data. Binding constants along with enthalpy, entropy, and free energy of binding are given in Table 1. The endothermic nature of Ca^{2+} displacement by Yb^{3+} is obvious considering the fact that the hydration enthalpy of Yb^{3+} is higher than Ca^{2+} (Marcus 1985). This implies that energy released on Ca^{2+} hydration is not sufficient to break the Yb^{3+} hydration shell to bind to the protein, resulting in heat being absorbed from the solution. The binding constants represent the following displacement reaction:

Here, n represents the number of Ca^{2+} bound to the protein ($n = 1.4$) and m and k represent the number of water molecules in the first hydration sphere of Yb^{3+} and Ca^{2+} , respectively. As discussed below, assignment of various binding constants to their respective metal binding sites in the protein has been carried out in concert with the NMR observations.

Discussion

EF-hand proteins maintain specificity for Ca^{2+} against its strong competitor Mg^{2+} , which is present in 1000-fold excess inside the cell. In the past, binding affinity of Ca^{2+} in EF-hand proteins has been shown to be largely determined by the amino acid composition of Ca^{2+} -binding loops (Mardsen et al. 1988; Waltersson et al. 1993; Wu and Reid 1997), although there have been few instances in which the helices flanking Ca^{2+} -binding loops also have been shown to influence the binding affinity (Sharma et al. 1997). The thermodynamics study of Ca^{2+} -binding in *EhCaBP* carried

Figure 6. Plot of normalized volumes of original as well as pseudocontact shifted (PCS) cross peaks for A118 and G122 belonging to Site IV as a function of the metal:protein ratio. The integral volumes of the pseudocontact shifted cross peak of G122 and A118 have been normalized with respect to the volume of their original cross peaks at a metal:protein ratio of 0.

Figure 7. (A) A calorimetric titration of 1.2- μL aliquots of 20 mM YbCl_3 solution into 0.24 mM Ca^{2+} -loaded *EhCaBP* at 308 K. (B) Plot of kcal/mole of heat absorbed/released per injection of YbCl_3 as a function of the metal:protein ratio at 308 K and the best least-squares fit of the data to binding models described in the text.

out earlier showed that IV site has the highest affinity for Ca^{2+} (Mardsen et al. 1988). On the other hand, the same study showed that there are two low-affinity sites for Ca^{2+} , both of which are exothermic in nature and two high affinity sites for Ca^{2+} , of which one is endothermic and one is exothermic. It also revealed that both the N-terminal Sites I and II in *EhCaBP* bind Ca^{2+} 100-fold less strongly compared to Site IV. In the present study, we rationalize the

binding affinity of Yb^{3+} to *EhCaBP*. We restrict the discussion to the Gly markers present at the sixth position of each of the four Ca^{2+} -binding loops of *EhCaBP*, although intensity profiles of other residues (shown in Figs. 3–6) have also been considered in the analyses. It is also assumed throughout the study that the two domains in *EhCaBP* do not show any interdomain cooperativity, and hence, bind metal independently.

As evident from Scheme 1, the third Ca^{2+} -binding loop (Site III) in *EhCaBP* possesses four charged residues (D85, D87, D89, and E96) that coordinate to Ca^{2+} as opposed to only three in the other three loops (I, II, and IV). Hence, Site III is expected to be the most preferred site for tripositive Yb^{3+} , in *EhCaBP* (Biekofsky et al. 1999). Also, out of the four Ca^{2+} -binding loops present in *EhCaBP*, the third loop structurally diverges the most from the corresponding loops of its homologous proteins, CaM and TnC (Atreya et al. 2001; Fig. 8). Such a divergence of the third Ca^{2+} -binding loop of *EhCaBP* could be attributed to the presence of a Tyr residue at position –4 with respect to the third loop, instead of a highly conserved Phe (Atreya et al. 2001). Previously, it has been shown that, in a given domain containing a pair of EF-hands, Phe at –4 position with respect to the first EF-hand loop (F[–4]) interacts with yet another conserved Phe at the 13th position of the second EF-hand loop (F[13]), such that their respective aromatic rings are oriented perpendicular to each other (Rashidi et al. 1999; Fig. 9). Such an orientation results in their $\text{C}^\alpha\text{--C}^\alpha$ distance of 7–8 Å (Rashidi et al. 1999). Further, proteins that lack such Phe–Phe interactions due to the absence of F(–4) or/and F(13), are found to have lower affinity for Ca^{2+} (Chandra et al. 1994; Ohya and Botstein 1994). In both CaM and TnC and in the N-terminal domain of *EhCaBP*, the respective Phe aromatic rings are oriented perpendicular to each other, with their $\text{C}^\alpha\text{--C}^\alpha$ distances in the range of 7–8 Å (Fig. 9). However, in the C-terminal domain of *EhCaBP*, the aromatic ring of Tyr 81 faces that of Phe 129 almost in a parallel orientation, resulting in their $\text{C}^\alpha\text{--C}^\alpha$ distance of 10.3 Å (Atreya et al. 2001). This causes the divergence of the third loop of *EhCaBP* compared to the corresponding loops of CaM and TnC (Fig. 8). Thus, the preference for Yb^{3+} to Site III can be attributed to both its higher negative charge compared to the other Ca^{2+} -binding loops in the protein and to

Table 1. Thermodynamic parameters obtained from Yb^{3+} titration of Ca^{2+} loaded-CaBP using ITC

Macroscopic binding constants (M^{-1})	ΔH (kJ/mole)	ΔG^a (kJ/mole)	$T\Delta S$ (kJ/mole)	Microscopic binding constants to Yb^{3+} sites in <i>EhCaBP</i>	Binding constants used in simulations (M^{-1})
$K_3 = (1.55 \pm 0.45) \times 10^4$	188.7 ± 87.6	–25.88	214.6	k_{II} (N-term)	$K_3 = (1.55) \times 10^4$
$K_2 = (2.46 \pm 1.40) \times 10^4$	117.1 ± 10.4	–24.69	141.8	$k_{\text{I,II}}$ (N-term)	$K_2 = (2.46) \times 10^4$
$K_1 = (1.25 \pm 0.38) \times 10^5$	41.8 ± 1.1	–30.04	71.8	k_{III} (C-term)	$K_1 = (4.50) \times 10^5$
$K_4 = (1.78 \pm 1.20) \times 10^3$	-210.5 ± 96.2	–19.16	–191.3	$k_{\text{IV,III}}$ (C-term)	$K_4 = (1.78) \times 10^3$

^a $\Delta G = -RT \ln(K)$.

Figure 8. Superposition of four Ca^{2+} -binding loops of *EhCaBP* (shown in blue) with the corresponding loops of *CaM* (red) and *TnC* (dark green). Both X-ray derived and NMR-derived structures of *CaM* (PDB code: 1CMG and 1CLL) and *TnC* (PDB codes 1TNX and 1TN4) are used in the comparison. The loops are numbered in the order from N-terminus to the C-terminus of the individual proteins. Thus, loops 1 and 2 belong to the N-terminal domains, while loops 3 and 4 belong to the C-terminal domains.

the presence of a Tyr at -4 position in the loop. Such a hypothesis is consistent with the NMR data, which show a rapid decrease in intensity of G90 amide cross peak during the initial stages of titration when the metal:protein ratio is increased from 0 to 1. Simultaneously, the pseudocontact shifted peak of G122 ($\delta^1_{\text{H}}^{\text{N}} = 9.3$ ppm) increases in its integral volume and reaches maximum at a little over metal:protein ratio of 1 (Figs. 1, 4, 6). We can therefore conclude that Yb^{3+} first displaces Ca^{2+} from the Ca^{2+} -binding Site III in *EhCaBP*, which results in a pseudocontact shift of residues belonging to Site IV.

Once the Site III is filled, the Yb^{3+} is left with two sites in the N-terminal domain and one more in the C-terminal domain. The simultaneous decline in the volumes of G15 and G51 cross peaks belonging to Sites I and II, respectively, and the appearance of pseudocontact shifted peaks for residues belonging to Site I alone (Figs. 1, 5), reveals that Yb^{3+} displaces Ca^{2+} preferentially in Site II. This results in the formation of $(\text{Ca}^{2+})(\text{I})-(\text{Yb}^{3+})(\text{II})-(\text{Yb}^{3+})(\text{III})-(\text{Ca}^{2+})(\text{IV})$ -*EhCaBP* species. The fact that no pseudocontact

shifted cross peaks were observed for residues in Site II, implies a complete absence of species such as $(\text{Yb}^{3+})(\text{I})-(\text{Ca}^{2+})(\text{II})-(\text{Yb}^{3+})(\text{III})-(\text{Ca}^{2+})(\text{IV})$ -*EhCaBP*. This gives rise to the question as to why Yb^{3+} preferentially displaces Ca^{2+} in Site II compared to Site I in the protein during this stage of titration. A closer examination of the respective Ca^{2+} -binding loop sequences (Scheme 1) reveals that there are five acidic residues (three Asp and two Glu) in the Ca^{2+} -binding loop II as opposed to four (two Asp and two Glu) in loop I. This results in a higher overall negative charge density in Site II compared to Site I, resulting in its higher preference for Yb^{3+} over the latter (against Ca^{2+}). Further, the Ca^{2+} -binding Site II has two consecutive aspartates (D46 and D48) at positions X and Y that directly coordinate to the metal, whereas Site I has D10 and N12 at the corresponding positions. This results in dentate–dentate repulsion between their negatively charged side chains of D46 and D48 in Site II, which can lower the Ca^{2+} -binding affinity more than the Yb^{3+} binding affinity, due to the higher positive charge of the latter. Similar observations have been reported earlier in studies involving different peptide sequences of Ca^{2+} -binding loops of *TnC* (Mardsen et al. 1988), wherein sequences with two Asp residues at consecutive Ca^{2+} coordinating positions such as X and Y, Y and Z, or X, Y, and Z showed lower Ca^{2+} -binding affinity

Figure 9. Orientation of the aromatic rings of F(-4)/Y(-4) and F(13) in N- and C-terminal domains of (A) Calmodulin (B) Troponin C, and (C) *EhCaBP*.

Figure 10. (A) Experimental and (B) simulated plots of intensity profiles of the original and pseudocontact shifted cross peaks of G15 and G122. The thermodynamic constants used in simulations are listed in Table 1 (last column; $K_2 > K_3$). (C) Simulated plots of the intensity profiles of original and pseudocontact shifted cross peaks of G15 and G122 obtained by interchanging the values of K_2 and K_3 values as 1.55×10^4 and $2.55 \times 10^4 \text{ M}^{-1}$, respectively.

compared to other peptide sequences lacking such consecutive Asp residues.

Further, the rate at which the pseudocontact shifted peaks of residues belonging to Site I increase in their integral volumes is relatively lower compared to the rate of decrease of the corresponding original peaks. This implies the coexistence of species $(\text{Yb}^{3+})\text{(I)}-(\text{Yb}^{3+})\text{(II)}-(\text{Yb}^{3+})\text{(III)}-(\text{Ca}^{2+})\text{(IV)}\text{-EhCaBP}$ (species A) and species $(\text{Ca}^{2+})\text{(I)}-(\text{Yb}^{3+})\text{(II)}-(\text{Yb}^{3+})\text{(III)}-(\text{Ca}^{2+})\text{(IV)}\text{-EhCaBP}$ (species B). Their relative populations can be determined from the knowledge of the ratio of integral volumes of cross peaks arising from the respective species.

Beyond a metal:protein ratio of 2.0, both the original and pseudocontact shifted cross peaks for residues in Site I show

a decline in their integral volumes (Figs. 1, 5A). This implies the filling of Yb^{3+} in Site I only in the species wherein Site II is already filled with Yb^{3+} . As observed, a net decrease in the pseudocontact shifted cross peak volumes implies that population of species B is relatively more compared to species A. Another important point to note is that, the reaction path that proceeds via the filling of Site I is completely ruled out in the present case, as we did not observe any pseudocontact shifted peaks for residues in Site II.

As far as the cooperativity is concerned, it is very well known that it exists between any two metal binding sites of adjacent EF-hands. Such cooperative binding of ions is the most important functional property of the calcium signaling pathways. So, it is not surprising that we also notice positive cooperativity during the process of our lanthanide titration discussed here. The most pertinent explanation for such cooperativity is that the binding of one metal facilitates the binding of the other metal ion, because the apo becomes half-holo, and causes the other site to loose its flexibility and become more structured. Such site-site communication in the EF-hand calcium binding proteins has been reported earlier (Maler et al. 2000). However, in the present case it does not go from apo to half-holo to holo. Instead, it is a displacement of Ca^{2+} ions by Yb^{3+} . To start with, both sites are already well structured and remain so even after the metal displacement. This observation, however, raises questions such as what is the origin of the positive cooperativity seen in the present case? And why should the second Yb^{3+} care to go to a site only when another Yb^{3+} , and not a Ca^{2+} , is in its neighboring site of a given pair of EF-hands? The answers to these questions lie in the difference in charges present in individual sites. When both sites are negatively charged there is an intrinsic repulsion between them, which is decreased when a metal ion binds to one of the sites, resulting in the formation of a short stretch of β -sheet, which in turn, facilitates the entry of the second metal ion. If a domain with a pair of EF-hands is already occupied by two Ca^{2+} ions, as is the case now, there could still be a slight excess of negative charge. Substitution of one of them with a tripositive lanthanide ion could decrease even more, or perhaps abolish, the residual electrostatic repulsion between the two loops, facilitating the entry of the second tripositive lanthanide ion. This argument is in line with the works reported earlier on the N-terminal domain of calbindin (Maler et al. 2000; Bertini et al. 2001c), where strong cooperativity was observed. When cerium ions occupy both the sites of the domain, the intermetal distance is reported to be sizably shorter than that observed between the two Ca^{2+} ions in the native protein.

As mentioned earlier, EF-hand proteins maintain specificity for Ca^{2+} against its strong competitor Mg^{2+} inside the cell. Hence, it could also be the same case with tripositive lanthanide ions against Mg^{2+} .

Coming to the ITC data, it yields four macroscopic binding constants, K_1 , K_2 , K_3 , and K_4 , as shown in Table 1, which can be now assigned to the Ca^{2+} displacement by Yb^{3+} from the respective Ca^{2+} -binding sites in *EhCaBP*.

Up to a metal:protein ratio of 1.0, Site III is predominantly filled with Yb^{3+} , leaving Sites I, II, and IV with Ca^{2+} . Thereafter, Sites I and II are filled completely at a metal:protein ratio slightly above 3.0. Further, it is interesting to note that, Site IV is left with 30% Ca^{2+} even when we go beyond a metal:protein ratio of 4.0. This implies that binding constants for Yb^{3+} in Sites III and IV of *EhCaBP* differ by a large factor, resulting in the sequential displacement of Ca^{2+} in the C-terminal domain. In the light of such observations, we can ascribe the highest binding constant (K_1) obtained in ITC to the Ca^{2+} displacement by Yb^{3+} in Site III (k_{III}), while the lowest (K_4) to the similar process in Site IV. It is important to note that K_4 is the binding constant of Yb^{3+} for Site IV when Yb^{3+} is already bound to Site III ($k_{\text{IV,III}}$). The assignment K_4 to Site IV is in line with the thermodynamics study of Ca^{2+} -binding in *EhCaBP* carried out earlier, which showed that Site IV has the highest affinity for Ca^{2+} in *EhCaBP* (Gopal et al. 1997). In the present study, the remaining two macroscopic binding constants, K_2 and K_3 obtained using ITC (Table 1) can be assigned to the Ca^{2+} displacement by Yb^{3+} in Sites I and II in the N-terminal domain. As discussed above, during the course of titration wherein the metal:protein ratio is changed from 1 to 3, NMR studies indicate that Yb^{3+} preferentially displaces Ca^{2+} in Site II. Further, at a metal:protein ratio of 2.0, the population of species B is about 45% and that of species A is about 25% of the total species present. The presence of species B in excess to that of species A implies that $k_{\text{I,II}} > k_{\text{II}}$. Thus, out of K_2 and K_3 , the smaller one (K_3) can be assigned to the Ca^{2+} displacement by Yb^{3+} in Site II (k_{II}); K_2 then represents the equilibrium constant of Ca^{2+} displacement by Yb^{3+} in Site I, when Yb^{2+} is already bound in its neighboring Site II ($k_{\text{I,II}}$).

To verify whether the above assignments of thermodynamic constants to the various reactions are correct, plots of normalized volumes of cross peaks (Figs. 2, 5, 6) were back

calculated using the equilibrium constants obtained from ITC (Table 1, last column). The reactions considered in the simulations are given in the Electronic Supplemental Material.

The experimental and simulated plots of intensity profiles are shown in Figure 10 for the original and pseudocontact shifted cross peaks of G15 and G122. As evident from the figure, simulated plots (Fig. 10B) obtained using the thermodynamic constants given in Table 1 (last column) are almost identical to the intensity profiles obtained for various species based on NMR titration data (Fig. 10A). The values of the thermodynamic constants used in the simulation are same as the experimentally observed ones, except for K_1 , which is found to be three times higher. To verify the assignments of K_2 and K_3 , simulated plots of intensity profiles were generated by interchanging the values of K_2 and K_3 . This is shown in Figure 10C. A lower value for K_2 compared to K_3 results in 40% population of the pseudocontact shifted cross peak of G15 at a metal:protein ratio 2. However, NMR data indicates about 25% population of this species at this metal:protein ratio (Fig. 5). Thus, a higher value of K_2 compared to K_3 (as given in Table 1) more appropriately describes the system.

Using the NMR and ITC data in concert, one can arrive at the following scheme of reaction for Ca^{2+} displacement by Yb^{3+} in *EhCaBP* that is most consistent with both the experimental data. In Scheme 2, black color sites indicate metal-binding sites completely filled with $\text{Ca}^{2+}/\text{Yb}^{3+}$, whereas those colored in gray indicate partial $\text{Ca}^{2+}/\text{Yb}^{2+}$ filling.

In conclusion, NMR and thermodynamics data conclusively show that Yb^{3+} has the highest preference for Ca^{2+} -binding Site III in *EhCaBP* compared to the other sites in the protein. This has been attributed to (1) the presence of a higher number of negatively charged residues in the third site, which are stabilized by Yb^{3+} , and (2) the presence of a Tyr at the -4 position in the third Ca^{2+} -binding loop, instead of a highly conserved Phe, which results in a relatively more open conformation of the loop. Thermodynamics studies reveal a 10–100-fold higher equilibrium constant for dis-

Scheme 2

placement of Ca^{2+} by Yb^{3+} at Site III compared to the rest of the sites. Further, dentate–dentate repulsion between charged residues in Site II in the N-terminal domain results in a preferential Ca^{2+} displacement by Yb^{3+} in Site II compared to Site I.

Finally, studies such as this will help in unraveling the number of Ca^{2+} -binding sites in a protein. It assumes importance for EF-hand proteins such as neuronal Ca^{2+} sensor 1 (NCS-1), wherein an exact number of Ca^{2+} -binding sites in the protein have not yet been resolved unambiguously (Cox et al. 1994; Ames et al. 2000). Moreover, in multidomain proteins, if we can observe pseudocontact shifts in one domain due to the filling of Ln^{3+} in the other, then such shifts help in fixing the interdomain orientations. Efforts are on in this direction to define the relative orientation of the two domains in *EhCaBP*.

Materials and methods

Protein sample preparation

The plasmid encoding *EhCaBP* was transformed into *Escherichia coli* BL21(DE3) cells. The protocol described earlier (Sahu et al. 1990a; Atreya et al. 2001) was used for overexpression of *EhCaBP* in a minimal (M9) medium. In the present study, two samples were prepared: (1) [$u\text{-}^{15}\text{N}$] and (2) [ALK]- ^{15}N -labeled (Ca^{2+})₄-*EhCaBP* (holo) samples. The details of growth condition and purification procedure have been described earlier (Sahu et al. 1999a; Atreya et al. 2001). The final step in the purification of *EhCaBP* involves the elution of protein from the anion exchange column using 10 mM CaCl_2 . Excess of Ca^{2+} in the protein sample was removed using a 3-kD cutoff Amicon centricon unit, by washing the protein solution six times with deionized water followed by three times with 0.1 M KCl solution containing 10% $^2\text{H}_2\text{O}$ and pH 6.0, and finally concentrating it to 0.6 mL for NMR studies. The protein concentration was estimated as 1.2 mM (~12.5 mg of protein in 0.6 mL) by measuring the absorbance at 280 nm ($\epsilon_{280} = 5120 \text{ M}^{-1} \text{ cm}^{-1}$) on a Varian Inc. Cary 50 scan spectrophotometer. NMR experiments were performed at 35°C on 0.6 mL of ~1 mM protein samples (pH 6.0), in a mixed solvent of 90% H_2O and 10% $^2\text{H}_2\text{O}$.

Yb^{3+} titration of [$u\text{-}^{15}\text{N}$] and [ALK]- ^{15}N -labeled (Ca^{2+})₄-*EhCaBP*

NMR experiments on *EhCaBP* were carried out in the form of a titration. The displacement of Ca^{2+} in the protein by Yb^{3+} was achieved by adding small aliquots from a 20 mM stock solution of YbCl_3 to the protein solution. To start with, an aliquot of YbCl_3 from a 0.02 M stock solution corresponding to a metal:protein ratio of 0.1 was taken each time and added directly to the protein solution in the NMR sample tube. The sample was mixed thoroughly before transferring it to the 600/700 MHz magnet. As the metal:protein ratio reached 0.5, large changes were observed in the HSQC spectrum and, hence, further titration was carried out in smaller steps of 0.05 metal:protein ratios. From a metal:protein ratio of 2.0 to 4.6, titrations were carried out in larger step sizes (at a variable metal:protein ratio of 0.1, 0.2, and 0.4). This resulted in

a total number of 47 titration points. The change in pH on addition of the lanthanide salt was found to be negligible.

NMR experiments

NMR experiments were recorded either on a Bruker Avance 700 MHz NMR spectrometer (CERM) or Varian Unity⁺ 600 MHz NMR spectrometer (NFMR), both equipped with pulsed field gradient units and triple resonance probes with actively shielded Z-gradients. Sensitivity enhanced 2D [^{15}N - ^1H] HSQC spectrum using pulsed field gradients for coherence selection (Kay et al. 1992) was acquired during each step of the titration. During the entire course of titration, the sample was found to be stable and soluble. ^1H chemical shift calibrations were carried out with respect to the methyl signal (at 0.0 ppm) of 3-(trimethylsilyl)[3,3,2,2- ^2H] propionate- d_4 (TSP), which has been used as an external reference. ^{15}N chemical shifts were referenced with respect to an external standard of ^{15}N -labeled ammonium chloride (2.9 M in 1 M HCl). The ^1H and ^{15}N chemical shifts published earlier (Sahu et al. 1999a, 1999b) were used to identify individual cross peaks in the 2D [^{15}N - ^1H] HSQC spectrum of *EhCaBP*.

ITC measurements and analyses

ITC measurements were performed with a Microcal Omega titration calorimeter. Samples were centrifuged and degassed prior to the titration and examined for precipitation, if any, after the titration. A typical titration consisted of injecting 1.2 μL aliquots of 20 mM YbCl_3 solution into 0.24 mM (1 mL) of the protein solution after every 3.5 min to ensure that the titration peak returned to the baseline prior to the next injection. A total of 31 injections were carried out. No precipitation was observed during the titration. Aliquots of more concentrated ligand solution were injected into the buffer solution (without the protein) in a separate ITC run, to subtract the heat of dilution. All experiments were repeated at least twice with different protein and ligand concentrations to establish reproducibility of the data. The ITC data were analyzed using the software ORIGIN (supplied with Omega Microcalorimeter). The amount of heat released per addition of the titrant was fitted to two/four or sequential/independent binding sites on the protein as given by Wisemen et al. (1989).

Considering a two-metal binding site system depicted in Scheme 3 (e.g., N- or C-terminal domain of *EhCaBP*), a situation can arise in which the values of the microscopic binding constants k_I and k_{II} for the two sites under consideration are widely different (one of them might be 100–1000-fold larger than the other). In such a situation, the protein will exhibit a step-wise or sequential binding/displacement of Ca^{2+} .

$$K_I = k_I + k_{II}; K_I K_{II} = k_I k_{II,I} = k_{II} k_{I,II}$$

Scheme 3

For example, if k_{II} is much larger than k_I , nearly all protein molecules in the solution will have Ca^{2+} bound in the second site, before both the sites are filled with Ca^{2+} . Thus, the macroscopic binding constants K_1 and K_2 (see above) can be assigned to k_{II} and $k_{I,II}$, respectively. Further, if k_{II} and $k_{I,II}$ are comparable, species in which only the second site is filled (arising due to k_{II}) and the species in which both the sites are filled (arising due to $k_{I,II}$) coexist. Their relative ratio is dictated by k_{II} and $k_{I,II}$. This situation is precisely illustrated in Scheme 3 above, where the thick lines represent the observed reaction pathway (i.e., $k_{II} \gg k_I$) for the system, and the dashed lines represent the forbidden path (see Results). Here, M represents the bound metal ion.

ITC experiments yield only the values of the macroscopic binding constant K_1 and K_2 . However, if $k_{II} \gg k_I$, the reaction path shown as dashed lines in Scheme 3 is completely ruled out and the macroscopic binding constants K_1 and K_2 essentially coincide with the microscopic binding constants k_{II} and $k_{I,II}$. Further, the values of k_{II} and $k_{I,II}$ are reflected in the relative populations of species A and B shown in Scheme 3. If k_{II} is much larger than $k_{I,II}$ (say 100-fold), then the population of species A will be largely predominant until all of the Site II is filled with the metal. On the other hand, if k_{II} and $k_{I,II}$ are comparable, both species A and B coexist and their relative population can then be determined by the ratio: $k_{II}/k_{I,II}$.

Electronic supplemental material

The reactions considered for simulating the intensity profiles of cross peak volumes in *EhCaBP*. Based on the reactions, the concentration of various experimentally observed species are derived using thermodynamic constants obtained from ITC data.

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