Chapter 2

Divers Models of Divalent Cation Interaction to Calcium-Binding Proteins: Techniques and Anthology

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Abstract

Intracellular Ca^{2+}-binding proteins (CaBPs) are sensors of the calcium signal and several of them even shape the signal. Most of them are equipped with at least two EF-hand motifs designed to bind Ca^{2+}. Their affinities are very variable, can display cooperative effects, and can be modulated by physiological Mg^{2+} concentrations. These binding phenomena are monitored by four major techniques: equilibrium dialysis, fluorimetry with fluorescent Ca^{2+} indicators, flow dialysis, and isothermal titration calorimetry. In the last quarter of the twentieth century reports on the ion-binding characteristics of several abundant wild-type CaBPs were published. With the advent of recombinant CaBPs it became possible to determine these properties on previously inaccessible proteins. Here I report on studies by our group carried out in the last decade on eight families of recombinant CaBPs, their mutants, or truncated domains. Moreover this chapter deals with the currently used methods for quantifying the binding of Ca^{2+} and Mg^{2+} to CaBPs.

Key words: Equilibrium dialysis, Fluorescent Ca^{2+} indicators, Flow dialysis, Isothermal titration calorimetry, Penta-EF-hand proteins, S100 proteins, Invertebrate-specific CaBPs, Centrins, NOX5

1. Introduction

A rise in cytosolic Ca^{2+} concentration is used as a universal signaling mechanism to control very diverse biological processes, such as exocytosis, contraction, cell growth, and cell death. It consists of an oscillating and transient increase of the mean Ca^{2+} concentration from 100 nM to 0.5–2 μM during stimulation (1). It is noteworthy that at the surface of different membranes cytosolic microdomains build up in which Ca^{2+} reaches a few hundred μM (2). The intracellular Ca^{2+}-binding proteins (CaBPs) have affinities which are fine-tuned to handle the intracellular Ca^{2+} signal. The

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affinities of CaBPs for Ca^{2+} vary considerably; moreover for a given protein the affinity for Ca^{2+} can be strongly affected by Mg^{2+}; for instance in the absence of Mg^{2+} parvalbumins display affinity constants for Ca^{2+} (K_{Ca}) of 10^9 to 10^7 M^{-1} which are decreased to 10^6 M^{-1} in the presence of millimolar concentrations of Mg^{2+} (3). On the other hand calmodulin shows affinity constants of the order of 10^5 M^{-1} and these values are nearly not affected by Mg^{2+} (4). Some CaBPs show much lower affinities: for instance several S100 proteins display K_{Ca} values of 3 to 8×10^3 M^{-1} (5). For such proteins the importance of the microdomains becomes evident. Also the interaction of CaBPs with target proteins may dramatically alter their affinity for Ca^{2+} (6). Moreover in the case of calmodulin the rather low affinity is compensated by its high cellular concentration and huge excess over the target enzymes (7).

Anthology of Ca^{2+}-binding to CaBPs: A first anthology has been reported in 1996 (8) and comprised calmodulin, troponin C, calbindin D-9k, parvalbumin and oncomodulin, sarcoplasmic Ca^{2+}-binding proteins (SCPs), and neuron-specific Ca^{2+}-binding proteins (NCS). Below are summarized more recently studied (in the present century) families of CaBPs, with emphasis on what they have added to our knowledge on divalent cation interaction to EF-hand motifs. By convention the term K_{dCa} (dissociation constant for Ca^{2+}) will be used when the sites in a CaBP do not display cooperative binding, while $[Ca^{2+}]_{0.5}$ will be used in cooperative systems.

1.1. Apoptosis-Linked Protein ALG2

ALG-2 encodes a dimeric CaBP of the penta EF-hand family that promotes Ca^{2+}-dependent apoptosis (9). We reported that cloned ALG-2 cDNA from mouse liver mRNA contained two types of sequences (10). One (named ALG-2,5) corresponded to the sequence reported before; the second (named ALG-2,1) was six nucleotides shorter, and the corresponding protein lacked the amino acid residues Gly121 and Phe122. Both transcripts are present in mouse tissues in the same 2:1 molar ratio. The amino acid sequence shows 5 EF-hands but only EF-I and EF-III are canonical Ca^{2+}-binding sites (9). Contrary to the metal-free form, in the presence of Ca^{2+} ALG-2 is completely insoluble and 0.5% Tween must be added in order to perform the Ca^{2+}-binding experiments. Both isoforms possess two sites with high affinity for Ca^{2+} ($K_{dCa} = 1.2$ μM for ALG-2,5 and 3.1 μM for ALG-2,1) and one site of much lower affinity (K_{dCa} ca 300 μM). All the sites are Ca^{2+} specific, i.e., 2 mM Mg^{2+} does not affect the binding isotherms. The crystal structure of ALG-2,5 shows bound Ca^{2+} in EF-I, EF-III, and EF-V (11). EF-V is also a dimerization domain, as in most other penta EF-hand proteins. One target of ALG-2,5 is Alix, but the shorter isoform ALG-2,1 does not interact with this target. Thus the minor sequence difference, which occurs in the linker between EF-III and EF-IV, affects the function of the proteins. In human uveal melanoma ALG-2,5 is down regulated and mutations

in the EF-hand revealed that EF-I and EF-III are the high-affinity sites and the binding of Ca^{2+} to both EF-I and EF-III is necessary for interaction with Alix (12).

1.2. Grancalcin

Grancalcin is a penta-EF-hand protein especially abundant in human neutrophils. Native and recombinant grancalcin always exist as a dimer regardless of the Ca^{2+} load (13). Metal-free grancalcin is fully soluble and in Ca^{2+}-containing buffers the protein is partly soluble, especially at micromolar concentrations; in the presence of 25 mM octylglycoside the protein is soluble even at 10 μM Ca^{2+}. From the amino acid sequence one can predict that, as for ALG-2, EF-I and EF-III are canonical Ca^{2+}-binding sites, whereas EF-II, EF-IV, and EF-V are abortive sites. As in other penta-EF-hand proteins EF-V is the dimerization module. The protein binds two Ca^{2+} with moderate affinity, with a K_{dCa} of 25 μM in the presence of octylglycoside and 83 μM in the absence of the detergent. The first Ca^{2+} seems to bind to EF-III without inducing any conformational change (14), whereas the binding of Ca^{2+} to the first EF-hand leads to pronounced changes in the Trp environment. In neutrophils the target of grancalcin is L-plastin, a leukocyte-specific actin-bundling protein, a mosaic protein containing two EF-hands, one calmodulin-binding domain, and two actin-binding domains. Ca^{2+} regulates the interaction in a negative manner, i.e., in the presence of EGTA the proteins interact and upon addition of an excess of Ca^{2+} the complex dissociates.

1.3. Calmodulin-Like Skin Protein

Calmodulin-like skin protein (CLSP), a 15.9 kDa CaBP specifically expressed in differentiated human keratinocytes (15), displays 52% sequence identity to calmodulin and possesses four EF-hands. The protein binds four Ca^{2+} ions at two pairs of sites with $[Ca^{2+}]_{0.5}$ of 1.2 (with very strong positive cooperativity: $n_H = 1.99$) and 150 μM (without cooperativity). Mg^{2+} at millimolar concentrations strongly decreases the affinity for Ca^{2+} of the two high-affinity sites, but has no effect on the low-affinity sites. The protein can also bind two Mg^{2+} ($K_{dMg} = 57$ μM) at the sites of high Ca^{2+} affinity (16). Thus, as in fast skeletal muscle troponin C, CLSP possesses two high-affinity Ca^{2+}/Mg^{2+} mixed sites and two low-affinity Ca^{2+}-specific sites. Studies on the isolated N- and C-terminal half domains of CLSP revealed that, in contrast to the case of TNC, the high-affinity Ca^{2+}–Mg^{2+} mixed sites reside in the N-terminal half. The binding of Ca^{2+} to the pairs of sites in each half, respectively, leads to the exposure of two hydrophobic patches, which are of equal quality as probed with the hydrophobic fluorescent TNS (see Note 5) and are directly governed by the Ca^{2+} affinity of that domain.

1.4. Calretinin

Calretinin is an abundant neuronal CaBP containing six EF-hand motifs and closely related to calbindin-D28k. In the cochlea it likely provides a strong, fast, and mobile buffer to cope with the

unusually high, localized Ca^{2+} spikes. Calretinin-22k is a splice product of calretinin (17 kDa) found specifically in cancer cells, and possesses four EF-hands (17). Calretinin binds four Ca^{2+} ions with high affinity ($[Ca^{2+}]_{0.5} = 2$ μM; $n_H = 1.3$) and one Ca^{2+} with much lower affinity ($[Ca^{2+}]_{0.5} = 50$ μM). Calretinin-22k binds three Ca^{2+} with high affinity ($[Ca^{2+}]_{0.5} = 1.2$ μM; $n_H = 1.3$) and one Ca^{2+} with low affinity (K_{dCa} above 1 mM). Since EF-VI shows a noncanonical amino acid sequence, it is likely that EF-V, which is paired to EF-VI, is the low-affinity site in both proteins. All the sites are of the Ca^{2+}-specific type. The secondary structure is not significantly altered upon the binding of Ca^{2+}, but Ca^{2+} induces changes in the microenvironment of Trp and leads to the exposure of a hydrophobic patch on the surface of both proteins (18).

1.5. S100 Proteins

S100 proteins (soluble in 100% ammonium sulfate) are dimeric proteins of ca 20 kDa. Each monomer contains 2 EF-hands: the C-terminal EF-hand displays the canonical sequence, whereas the N-terminal EF-hand, consisting of 14 amino acids, is specific for S100 proteins. To date, some 21 different proteins, which display various degrees of amino acid sequence homology, have been assigned to the S100 protein family (19). The protein contains a dimerization domain, an X-type four-helix bundle consisting of the N-terminal helix of EF-I and the C-terminal helix of EF-II. The conformation of this socle is insensitive to Ca^{2+}. Binding of Ca^{2+} induces small changes in the loop of the second EF-hand and a reorientation of the third helix. This swing-out movement on both sides of the protein dimer leads to unmasking of hydrophobic residues, thus forming two symmetric hydrophobic patches. The different S100 proteins interact in a Ca^{2+}-dependent manner with a large number of target proteins. Since a previous review covered the studies of the last quarter of the twentieth century (5), this overview deals with the ion-binding properties of S100 proteins reported in the last decade: S100A5, S100A13, and S100A16.

S100A5 is expressed in some restricted regions containing neurons of the brain (20). The four Ca^{2+} in the homodimer bind with high affinity and positive cooperativity ($[Ca^{2+}]_{0.5} = 6$ μM; $n_H = 1.9$) and the sites are of the Ca^{2+}-specific type. The dimer of S100A5 also binds 2 Zn^{2+} ($[Zn^{2+}]_{0.5} = 2$ μM) and 4 Cu^{2+} ($[Cu^{2+}]_{0.5} = 3$ μM). In the monomer Zn^{2+} binds to a site different from the Ca^{2+} site, likely opposite to the two Ca^{2+} sites. In contrast Cu^{2+} seems to use the Ca^{2+} ligands. The secondary structure does not change upon binding of metal ions, but the microenvironment of the Tyr changes upon binding of Ca^{2+}, but not of Zn^{2+}.

S100A13 is expressed in different tissues, but often in a cell type-specific manner in these tissues; it seems to function in exocytosis since it is a target of anti-allergic drugs (21, 22). The protein dimer binds 4 Ca^{2+}, two with $[Ca^{2+}]_{0.5}$ of 8 μM ($n_H = 1.3$) and two with $[Ca^{2+}]_{0.5}$ of 400 μM ($n_H = 1.3$). Mg^{2+} (2 mM) has no effect on

the Ca^{2+}-binding isotherm. As for S100A5 Ca^{2+}-binding does not change the secondary structure, but it has a strong effect on the microenvironment of the single Trp. Surprisingly, S100A13 does not bind to phenyl-Sepharose, as do all other S100 proteins, and the Ca^{2+}-loaded protein does not influence the hydrophobic probe TNS, thus strongly suggesting that this Ca^{2+}-S100A13 isoform has no hydrophobic patch on its surface.

S100A16 is predominantly expressed in astrocytes in adult brain where it is present in the nucleus (concentrated in the nucleolus) and cytoplasm in vivo (23); in fact the intracellular free Ca^{2+} regulates the nuclear translocation of S100A16 in the sense that high intracellular Ca^{2+} levels induce nucleolar exit and nucleocytoplasmic transport and lowering the intracellular Ca^{2+} concentration leads to nuclear translocation. S100A16 binds only 2 Ca^{2+} ions per dimer with a K_{dCa} of 750 μM ($n_H = 1$) for the human protein and 430 μM for that of mouse. From the amino acid sequence it can be inferred that Ca^{2+} binds to the second EF-hand in each monomer. The mouse protein binds also 2 Zn^{2+} per dimer with an estimated K_{dZn} of 25 μM and, as for S100A5, likely at a different site than that for Ca^{2+}. Surprisingly Ca^{2+}-binding does not induce a hydrophobic patch on the surface of the mouse protein, whereas Zn^{2+} does so.

1.6. Invertebrate-Specific Ca^{2+}-Binding Proteins

A family of intracellular CaBPs was found to be spread over all forms of life, from bacteria to protochordates, in invertebrate muscle and brain, except in vertebrates. We called it the SCP family since for a long time only the proteins present in the sarcoplasm, i.e., in the supernatant after centrifugation of a muscle homogenate extracted in physiological ion salt concentrations, were known. They have all a very similar three-dimensional structure, which is characteristic, and unique for this family, and are involved in a variety of functions including intracellular Ca^{2+} buffering, bioluminescence, enzymatic activity, and associated learning. Members of this family are SCP, calerythrin (a Ca^{2+}-buffering protein in the gram-positive bacterium *Saccharopolyspora erythraea*), aequorin (a bioluminescent protein in the jellyfish *Aequorea aequorea*), and calexcitin found in the nervous tissues of insects and mollusks. The Ca^{2+}-binding properties of SCPs (considered to function only as intracellular Ca^{2+}-buffering proteins) of arthropods, annelids, protochordates mollusks, and insects have been described in the period 1975–1990 and have been reviewed (24).

Calerythrin possesses 4 EF-hands, but EF-II does not possess a canonical Ca^{2+}-binding loop. It displays three high-affinity Ca^{2+}-binding sites, two of which are strongly cooperative. The NMR solution structure (25) shows much similarity with sandworm SCP. Aequorin consists of a 22 kDa apoprotein, the hydrophobic prosthetic coelenterazine moiety, and an oxygen molecule. Aequorin binds three Ca^{2+} with high positive cooperativity and

subsequently undergoes a series of autocatalyzed reactions leading to the emission of a photon. The crystal structure (26) shows an overall conformation very similar to sandworm SCP, but, contrary to the latter, in the center of aequorin there is a cavity with a volume of 600 Å^3. The cavity is entirely filled with the substrate 2-hydroperoxycoelenterazine which is oxidatively decarboxylated to coelenteramide.

Calexcitin, the most recent member of the SCP family (27), plays a role in associative learning through inhibition of K^+ channels and activation of the ryanodine receptor, both in a Ca^{2+}-dependent manner (28). Calexcitin from the optic lobe of squid *Loligo pealei* has four EF-hands but EF-IV is clearly inactive. The sequence identity with amphioxus SCP is 31%. Calexcitin is monomeric and binds three Ca^{2+} ions with a $[Ca^{2+}]_{0.5}$ of 0.8 μM and n_H of 1.6, meaning pronounced cooperativity. Similar to be case of SCPs the relation between Ca^{2+} and Mg^{2+} is complex: the data can best be interpreted as follows: calexcitin possesses one Ca^{2+}-specific site and two Ca^{2+}/Mg^{2+} mixed sites. Of the mixed sites one is very sensitive to Mg^{2+}, whereas the other displays a weak Mg^{2+} effect (29). The thiol reactivity, circular dichroism, Trp fluorescence, thermal stability, and urea denaturation, all indicate that the protein displays three distinct structures based on the type of ion that is bound, as do the genuine SCPs. From hydrophobic probe binding it can be inferred that the metal-free protein has molten globule characteristics, the Ca^{2+}-form displays a hydrophobic grove, likely involved in protein–protein interaction, and the Mg^{2+} form is very compact without a hydrophobic patch. The three-dimensional structure at 1.8 Å resolution of calexcitin from *Loligo paelei* (30) displays a compact molecule with a very pronounced hydrophobic core and a scaffold similar to that of amphioxus SCP.

1.7. Centrins

Centrins are ubiquitous Ca^{2+}-binding proteins in higher plants, yeast, algae, invertebrates, and vertebrates, concentrated in microtubule-organizing centers (MTOCs), which are named spindle pole bodies in yeast, basal bodies in algae, and centrosomes in higher eukaryotes. Centrins are essential for cell division, for mRNA nuclear transport, and for nucleotide excision repair. Moreover in green algae centrin participates in a network of Ca^{2+}-sensitive fibers forming connections between them and with the nucleus. These fibers contract in a Ca^{2+}-dependent, ATP-independent manner and centrins seem to be the Ca^{2+} sensor for contraction. The molecular mechanism of centrin-mediated motility is actually not known, but a clear sign of progress was the discovery of a protein called Sfi1, containing a large number of similar sequence motifs, each capable to bind independently one centrin molecule (31). Salisbury (32) postulated a novel mechanism of MTOC duplication based on a "unit of duplication" requiring either C-terminal tail-to-tail homodimerization of Sfi1, thus

forming the spindle pole body in yeast, or oligomerization (a 9-mer) in green algae and likely in vertebrates, thus forming a cartwheel. Ca^{2+}-binding to the multiple centrins aligned on the Sfi1 fibers may cause changes in the length of these fibers. All these functions are centrin specific and not supported by calmodulin.

Centrins (ca 18 kDa) are structurally closely related to calmodulin with four EF-hands and structural independence of the two EF-hand domains. But, whereas calmodulin displays four Ca^{2+}-specific sites of almost the same affinity and no, or nearly no, effect of Mg^{2+}, the Ca^{2+}- and Mg^{2+}-binding properties are different, and even differ widely within the centrin family. Comparative sequence analysis allowed the establishment of a phylogenetic three that has four main subfamilies. *Chlamydomonas reinhardtii* (CrCen) is the prototype of the largest subfamily which comprises also human centrin 1 (HsCen1) and HsCen2. Yeast centrin (Cdc31) and HsCen3 form the next subfamily, while plant and *Paramecium* centrins define the two other subfamilies.

Human centrin 2 (HsCen2) is a homodimer and binds only one Ca^{2+} in each monomer with rather low affinity ($[Ca^{2+}]_{0.5} = 30\,\mu M$) and negative cooperativity ($n_H = 0.68$) (33). Mg^{2+} does not affect the Ca^{2+}-binding curve. In the amino acid sequence EF-IV is the only EF-hand with a canonical Ca^{2+}-binding loop. But in the presence of a model peptide (33) or in the isolated C-terminal half which possesses in addition helix D (F86-T94) at its N-terminus, called LC-HsCen2 (34), two bound Ca^{2+} ions are present per monomer; thus under certain conditions EF-III, which contains Thr in the +Z and Asn in the –Z position, can bind Ca^{2+}. The binding of Ca^{2+} induces a hydrophobic patch on the surface of HsCen2, only in the C-terminal half, consistent with the observation that the protein binds one mole of melittin, a model peptide for calmodulin interactions. HsCen1 is in several of these characteristics very similar to HsCen2 (not published observations).

Human centrin 3 (HsCen3) contains one high-affinity Ca^{2+}/Mg^{2+} mixed site ($[Ca^{2+}]_{0.5} = 3\,\mu M$, $[Mg^{2+}]_{0.5} = 10\,\mu M$) and two Ca^{2+}-specific sites of low affinity ($[Ca^{2+}]_{0.5} = 140\,\mu M$). The high-affinity site could be assigned to the N-terminal half of HsCen3, very likely to EF-I which contains an acid pair (35). Temperature denaturation, circular dichroism, and utilization of fluorescent hydrophobic probes allowed us to propose that the metal-free protein has molten globule characteristics and that the dication-bound forms are compact with a polar surface for the Mg^{2+} form and a hydrophobic exposed surface for the Ca^{2+} form.

Clamydomonas reinhardtii centrin (CrCen, also called caltractin) is a major component of the basal body connector and of the distal striated fiber that connects the basal bodies of contractile fibers in green algae and plays an essential role in flagellar excision. It has four functional EF-hands in two distinct and independent domains and may be the most ancestral of centrins since it

resembles the most to calmodulin. But, whereas calmodulin has four Ca^{2+}-specific sites of nearly identical affinity, CrCen possesses two high-affinity EF-hands $K_{dCa} = (1.2\ \mu M)$ in the N-terminal domain and two low-affinity sites ($[Ca^{2+}]_{0.5} = 160\ \mu M$) in the C-terminal domain (36). The effect of Mg^{2+} on these Ca^{2+}-binding properties has not been studied. A target peptide excised from the yeast protein Kar1 binds to each of the CrCen domains in a Ca^{2+}-dependent manner, with the C-terminal domain binding stronger than the N-terminal domain. Thus CrCen binds two Kar1p and can act as a bridging factor in MTOCs.

Scherffelia dubia centrin (SdCen) is, as CrCen, a component of basal body-associated contracting fibers in this green alga, and it resembles this latter centrin also in Ca^{2+}-binding (37). SdCen possesses two high-affinity Ca^{2+} sites ($[Ca^{2+}]_{0.5} = 3\ \mu M$) and one low-affinity site ($[Ca^{2+}]_{0.5} = 20\ \mu M$). In the presence of Mg^{2+} one of the high-affinity sites displays a fourfold lower affinity. Experiments with the isolated halves showed that the two high-affinity sites correspond to EF-I and EF-II and the low-affinity site likely is EF-IV. Ca^{2+}-binding to each half induces a hydrophobic patch with a sensitivity toward Ca^{2+} which is dictated by the affinity of Ca^{2+} for that domain. Each of the hydrophobic patches can bind a target peptide corresponding to one repeat of Sfi1 or of an identified consensus motif in the XPC protein which is involved in nuclear excision repair. As in the case of CrCen, the peptide interaction is stronger for the C-terminal domain. Thus SdCen is in many aspects similar to CrCen, except that it binds only one Ca^{2+} in the C-terminal domain.

Saccharomyces cerevisiae centrin (Cdc31) binds to yeast Sfi1, which contains 21 conserved repeats, each able to bind one molecule of centrin. The crystal structure of a Cdc31 complex with two or three repeats of Sfi1 shows Sfi1 as a long α-helix with the centrins wrapped around (38). As calmodulin, the centrin molecules are organized in two domains; the N-terminal domain is in a closed conformation (antiparallel EF-hand α-helices), whereas the C-terminal domain is in an open conformation (perpendicular EF-hand α-helices). Cdc31 binds Sfi1 via a deep hydrophobic cavity of the C-terminal domain, while its N-terminal domain has only limited contacts with Sfl1. Adjacent centrin molecules are rotated by 65° along the Sfi1 α-helix. Cdc31 contains one high-affinity Ca^{2+}/Mg^{2+} mixed site ($K_{dCa} = 0.3\ \mu M$; $K_{dMg} = 44\ \mu M$) and two low-affinity Ca^{2+}-specific sites ($[Ca^{2+}]_{0.5} = 28$ and $41\ \mu M$) (39). The high-affinity mixed site could be attributed to the N-terminal domain. Both Ca^{2+} and Mg^{2+} increase the α-helix content and stabilize the protein. In apo Cdc31 the N- and C-terminal domains are fused together, whereas in the Mg^{2+} state they are well separated as shown by the groove in the middle of the molecule. The affinity of Cdc31 for five different peptides excised from natural target proteins shows a Ca^{2+} and Mg^{2+} dependence and the peptides

occupy the central groove. Contrary to the green algae centrins, but similar to the human proteins, Cdc31 interacts with peptides in a 1:1 molecular ratio.

1.8. NADPH Oxidase 5

NADPH Oxidase 5 (NOX5) is a monomeric transmembrane Ca^{2+}-dependent oxidase with multiple domains (40): two heme groups attached to two of the six transmembrane α-helices, a long intracellular C-terminus with NADP- and FAD-binding sites, called *c*atalytic *deh*ydrogenase *d*omain (CDHD), and an intracellular N-terminus with four EF-hands called NOX5-EF. When NOX5 is activated it transports electrons from intracellular NADPH by means of FAD and the two heme groups to extracellular oxygen resulting in the formation of superoxide radicals. Contrary to the other NOX isoenzymes, which need regulatory subunits, NOX5 activation is initiated by the binding of Ca^{2+} to the N-terminal regulatory domain which interacts then directly with the catalytic C-terminal domain (41). Two short segments that represent the binding site of NOX5-EF have been identified (42); they are intrinsically autoinhibitory and the inhibition can be relieved by Ca^{2+}-dependent binding to NOX5-EF. In addition, in the presence of Ca^{2+}, calmodulin binds to the enzyme at a well-identified 19 residue long segment in the CDHD and this represents an additional sophistication in the regulation of superoxide production by NOX5 (43).

NOX5-EF possesses two pairs of EF-hands with a higher affinity ($K_{dCa} = 3.8$ μM) for the C-terminal pair than the N-terminal pair ($K_{dCa} = 17$ μM) and these affinities are not affected by Mg^{2+} (41); thus as in calmodulin they are of the Ca^{2+}-specific type. Interestingly whereas sites II, III, and IV have canonical sequences, site I displays a sequence typical of some sites in the PEF proteins sorcin, grancalcin, and the heavy and light chains of calpains, with Ala in the +X position providing its carbonyl oxygen and a water molecule oxygen in +Y. Upon binding of Ca^{2+} a hydrophobic patch is exposed on the surface of NOX5-EF, but only in the C-terminal pair. However conformational changes that affect the intrinsic fluorescence of Trp residues occur in both pairs of EF-hands.

2. Materials

2.1. Solutions

All solutions are prepared with ultrapure water bidistilled in a quartz apparatus to attain a conductivity of 10 mΩ cm. The standard working buffer in our studies is 50 mM Tris–HCl, pH 7.5, 150 mM KCl. Buffer solutions are decalcified by passage over a 2–15 cm column of Chelex-100 (Bio-Rad Laboratories).

Trichloroacetic acid is from Calbiochem. The contaminating Ca^{2+}, Mg^{2+}, or Zn^{2+} was measured by atomic absorption spectrophotometry (Perkin Elmer 2380).

2.2. Equilibrium Dialysis

Equlibrium dialysis of 1 ml protein solution was performed for 48 h in the cold room in Spectra/Por dialysis tubes (Spectrum Europe B. V.) versus 200 ml buffer in polypropylene bottles on a lab shaker with one buffer change after 24 h of dialysis.

2.3. Fluorescent Ion Indicators

Benzothiazole coumarin (BTC) and Mag-fura-2 are from Molecular Probes, Eugen, Oregon.

2.4. Flow Dialysis

Flow dialysis was performed in homemade flow dialysis cells with volumes of the upper and lower compartment of 500 µl and 50 µl, respectively (see Note 1). The flow rate was 1 ml/min, the volume of a fraction 0.4 ml. As tracer, 5 µM $^{45}Ca^{2+}$ (950 mBq/mg) were added to the upper compartment. The titration, done by a Microlab-2200 (Hamilton), consisted of additions every 2 min of 1–4 µl of the following stock solutions of Ca^{2+}: 0.63, 3.05, 9.58, 82.5 mM and the final chase was done with 1 µl of 1 M Ca^{2+}.

2.5. Isothermal Titration Calorimetry

Isothermal titrations were carried out at 30°C on a VP-ITC instrument (Microcal Inc Northampton, MA) in 50 mM Mops buffer, pH 7.4, 100 mM NaCl. A solution of 30 µM protein was titrated by automatic injections of 5–10 µl of 1 mM Ca^{2+}.

3. Methods

Since most methods require removal of the metal ions from the protein, a short first section is devoted to this topic.

3.1. Metal Removal from CaBPs

All the methods for metal binding described below require a strict control of the Ca^{2+} and Mg^{2+} levels and of the contamination of the buffers and protein solutions. Therefore it is nearly indispensable to have access to an atomic absorption spectrometer (AAS), called also flame-spectrometer. The natural contamination of solutions by Mg^{2+} is negligible (i.e., below 0.03 µM), but the Ca^{2+} contamination is several µM. Therefore all solutions used must be rendered "calcium-free" by treatment with Chelex 100 resin (Bio-Rad Laboratories). To reduce Ca^{2+} contamination all solutions must be kept in plastic containers, which have been soaked in 1 N HCl and rinsed with bidistilled water. In the second step Ca^{2+} must be removed from the CaBPs by methods which do not alter the binding properties. The softest protein decalcifications are done at neutral pH under non-denaturating conditions, but one can take advantage of the fact that CaBPs are surprisingly stable and easily

accommodate reversible denaturation. A panoply of fast (taking 1 h or less) methods is listed below.

1. After addition of 1–2 mM EDTA 1 ml of the concentrated protein solution is chromatographed on a 0.8–40 cm column of Sephadex G-25 equilibrated in the "Ca-free" working buffer (see Note 2). There is a risk that complexes of weak affinity are formed between the protein and EDTA, but likely they dissociate during the gel filtration.
2. Passage of the concentrated protein solution over a column of Chelex-100 resin or of EDTA-agarose equilibrated in the working buffer. It must be noted that the Ca^{2+} binding leads to proton exchange, thus necessitating strong pH buffers. In this method the protein is in fact not perfectly equilibrated in the working buffer.
3. Acidification of the protein solution to pH 2 followed by Sephadex G-25 chromatography in the working buffer.
4. Denaturation of the protein in 6 M guanidine-HCl at pH 8.0 followed by Sephadex G-25 chromatography in the working buffer.
5. Protein precipitation by addition of 3% trichloroacetic acid (TCA) and resolubilization at pH 8.0. The contaminating Ca^{2+} is monitored by AAS and eventually the precipitation is repeated At the end the dissolved protein is put on the Sephadex-25 column.

The last method is very efficient and has been applied successfully in my laboratory on dozens of proteins. In several cases we compared the Ca^{2+}-binding data for samples obtained by either the TCA method (numbered 5, above) or methods 1 and 2: no significant differences were detected. In contrast the Ca^{2+}-binding properties of some SCPs, CaVP, and certain mutants of parvalbumins are damaged by the TCA method.

3.2. Ca^{2+} Binding

The first question to be solved after one realizes that a given new protein contains EF-hand motifs is if it really binds Ca^{2+}. For this purpose qualitative methods exist such as the Ca^{2+}-dependent mobility shift in polyacrylamide gel electrophoreses (PAGE). Different optical methods such as fluorimetry on the intrinsic Trp or Tyr residues can be performed in most laboratories and lead to a valid diagnosis provided ion-binding leads to signal changes. But quantitative information, leading to the evaluation of the physiological significance of Ca^{2+}-binding to a protein, cannot be gathered by these methods. This section deals with the four most common methods to determine Ca^{2+}-binding isotherms.

3.2.1. Equilibrium Dialysis

In this method an equilibrium is established on both sides of a solute-permeable, but protein-impermeable, membrane (Spectra/

Por 3). In practice, aliquots of a protein solution (20–100 μM of binding sites) are equilibrated against a series of buffers of increasing ion (Ca^{2+}) concentrations. At the end the concentrations of protein and of total ligand are measured in both compartments. The difference in ligand concentration between both compartments corresponds to the bound ligand, which can be directly related to the protein concentration. Ion quantification can be done with AAS. A major complication is that it is impossible to decontaminate buffers of physiological ionic strength to free Ca^{2+} values lower than 0.2 μM. In order to attain free Ca^{2+} levels of 10^{-9} to 10^{-5} M, it is thus necessary to "clamp" the free Ca^{2+} by including 1 mM EGTA in the dialysis experiment. The free Ca^{2+} is then calculated using a computer program (WEBMAXC STANDARD version 2.10) of "speciation" taking into account the affinity constants of EGTA + Ca^{2+} and EGTA + H^+ and the total concentrations of EGTA and Ca^{2+}. Even with this complication the method is quite simple and needs minimal equipment. But three negative points must be mentioned: (1) The length (24 h) precludes determination on labile, sticky, or hydrolysis-susceptible proteins. (2) In the EGTA buffering system the free Ca^{2+} versus total Ca^{2+} relationship is not linear and lacks precision in the 10^{-7} to 2×10^{-5} M range, i.e., in the zone one usually wants to cover. (3) The workload is quite heavy and the number of manipulations requires maximal attention of the experimenter.

3.2.2. Fluorescent Ion Indicators

The principle of this method is competition for Ca^{2+} of the CaBP and an indicator whose binding parameters are well known and whose degree of saturation can be quantified exactly (44). To the mixture of protein and indicator increasing Ca^{2+} concentrations are added until the indicator is saturated (see Note 3). If the physical and optical properties of the indicator are the same in the presence and absence of protein, one can determine the free Ca^{2+} concentration by Eq. 1:

$$[Ca^{2+}] = \Delta s_i \,/\, (\Delta s_{max} - \Delta s_i) K_{indicator} \qquad (1)$$

where $[Ca^{2+}]$ is the concentration of free Ca^{2+}, Δs_i and Δs_{max} are the optical signal change at titration point i and at the end (maximal saturation of the chelator), respectively, and $K_{indicator}$ is the affinity constant of the indicator. The value of $K_{indicator}$ under the experimental conditions can be determined by a separate titration of the indicator alone. Protein-bound Ca^{2+} (Ca_{bound}) can then be calculated as follows:

$$Ca_{bound} = Ca_{Tot} - [Ca^{2+}] - \{K_{indicator} C_{indicator} [Ca^{2+}] \,/\, (1 + K_{indicator} [Ca^{2+}])\}, \qquad (2)$$

where Ca_{Tot} and $C_{indicator}$ are the concentration of added Ca^{2+} and of the indicator, respectively. If the protein concentration is in large excess over that of the indicator the last factor in Eq. 2 can be neglected.

Fluorescent probes are used at submicromolar concentrations while the protein concentration is 2–3 μM. In fluorescent titrations the spectra of the indicator often show an isosbestic point, a wavelength where no ligand-induced changes occur during the titration. The precision is much better when the signal at the wavelength of maximal fluorescence change is rationed to the signal at the isosbestic wavelength. An important consideration is the choice of the indicator with respect to its affinity for Ca^{2+}. Ideally $K_{indicator}$ should be slightly lower than that of the lowest affinity sites of the protein. Fortunately, many indicators have been developed by Molecular Probes with different affinities for Ca^{2+}, ranging from 10^7 to 10^4 M^{-1}. If a CaBP shows pronounced negative cooperativity or displays more than one site with markedly different affinities, the indicator method is not recommended. A last caveat is the selectivity of the indicator, especially if one wants to determine the Ca^{2+}-binding properties in the presence of physiological concentrations of Mg^{2+}. Ideally the Ca^{2+}/Mg^{2+} selectivity ratio should be higher than 10,000. Indicators with a low selectivity ratio can in fact be used to determine the Mg^{2+}-binding properties of a CaBP by similar Mg^{2+} titration experiments in the absence of Ca^{2+} (i.e., in the presence of EGTA).

3.2.3. Flow Dialysis

In this method an immobile solution (upper compartment) is separated from the lower compartment by a semipermeable membrane which retains the protein but not the small ligands; the lower chamber is continuously flushed with the working buffer (45). Flow dialysis is based in the principle that the amount of Ca^{2+} diffusing per unit of time from the upper compartment is proportional to the free Ca^{2+} in that compartment. If the Ca^{2+} pool in the upper compartment is tagged with $^{45}Ca^{2+}$, this rate can be measured by determining the concentration of the isotope in the effluent of the lower chamber (for commodity called here cpm_i), which is collected in a fraction collector. After perfusion with four times the volume of the lower chamber a steady state is reached for which the rates (dcpm/dt) of isotope entering and leaving the lower chamber are practically equal, as indicated by Eq. 3:

$$dcpm_i / dt = D[Ca^{2+}] - cpm_i(f / V) = 0, \qquad (3)$$

hence $[Ca^{2+}]_i = cpm_i\ (f/V.D)$,

where D is a constant depending on both the diffusion properties of Ca^{2+} and the geometry of the dialysis apparatus, f is the flow rate of buffer through the lower chamber, and V is the volume of this chamber. In essence, Eq. 3 indicates that after an equilibrium is reached, the concentration of isotope (cpm_i) in the effluent becomes a true measure of free $[Ca^{2+}]$ in the upper compartment. After each addition of unlabeled Ca^{2+} to the upper chamber, a new equilibrium is established and a new steady state of cpm_i is reached.

The parameters ($f/V.D$) are not determined directly; instead a large excess of cold Ca^{2+} is added at the end of the titration so that all Ca^{2+} and $^{45}Ca^{2+}$ can be considered as free. The corresponding cpm_{end} value is an index standing for 100% free [Ca^{2+}] and the ratio of cpm_i/cpm_{end} represents thus the fraction of free Ca^{2+} in the upper compartment. From the known total Ca^{2+} concentration at each increment the concentration of free and bound Ca^{2+} in the upper chamber is calculated. This data treatment is not complete enough since three factors are not negligible: (1) The dilution ($Fdil_i$, or dilution factor) in the upper compartment by the increments; (2) the losses ($Floss_i$, or loss factor); and (3) the Ca^{2+} (and tracer $^{45}Ca^{2+}$) contamination in the upper chamber at the start of the experiment. For this purpose the following additional parameters must be measured:

1. The initial Ca^{2+} contamination, $[Ca^{2+}]_{contam}$: After the cell is mounted, the upper chamber is filled with the protein solution and tracer $^{45}Ca^{2+}$ is added; then 250 µl is withdrawn to measure the Ca^{2+} concentration by AAS.
2. The initial (CPM_{init}) and final $^{45}Ca^{2+}$ counts (CPM_{end}) in the upper compartment: The ΔCPM factor, defined as $(CPM_{init} - CPM_{end})/CPM_{init}$, determines the total loss of Ca^{2+} in the upper chamber, which can vary substantially (from 10 to 20%) depending on the profile of free Ca^{2+} change in this chamber during the experiment. The fraction of free Ca^{2+}, $f_{[Ca2+]i}$, after increment i is as follows:

$$f_{[Ca2+]i} = (cpm_i Fdil_i Floss_i) / (cpm_{end} Fdil_{end} Floss_{end}), \qquad (4)$$

with $Fdil_i = Vol_i/Vol_{init}$ and $Fdil_{end} = Vol_{end}/Vol_{init}$ and $Floss_i = 1 + \Delta CPM \{\Sigma_{(i = 1 \text{ to } n)} cpm_i/\Sigma_{(i = 1 \text{ to } n)} cpm_i\}$.

The total Ca^{2+} concentration, $Catot_i$, after increment i is

$$\begin{aligned} Catot_i &= ([Ca^{2+}]_{contam.} + S[Ca^{2+}]_{increm.i}) / Fdil_i Catot_i \\ &= ([Ca^{2+}]_{contam.} + S[Ca^{2+}]_{increm.i}) / Fdil_i . \end{aligned} \qquad (5)$$

The free Ca^{2+} concentration corresponds to $f_{[Ca2+]i}$. $Catot_i$ and the protein-bound Ca^{2+} are then calculated by $Ca_{bound}^{2+} = Catot_i - [Ca^{2+}]$.

In practice the following experimental conditions are used for most of the CaBPs. The flow device (see Note 1) was mounted using Spectra/Por membranes, which were boiled twice: once in EDTA-alkalinized water and once in distilled water. The upper chamber contained 750 µl of a protein solution of 60 µM Ca^{2+}-binding sites. From this chamber 250 µl is withdrawn just after the addition of tracer $^{45}Ca^{2+}$. The lower chamber is perfused at a flow rate of 1 ml/min and the effluent is collected in fractions of 0.4 ml. Every 2-min increments of Ca^{2+} are added so that the total Ca^{2+} concentrations in the upper compartment vary according to an exponential scale until a value of 2–3 mM is attained. The samples

of the fraction collector are supplemented with scintillation liquid and counted to 98% precision. This method has allowed us to determine quite easily and precisely the Ca^{2+} affinity of a great number of proteins with K_{Ca} values varying from 5×10^7 to 2×10^3 M^{-1}. The precision is greatly improved by automation (robot) of the pipetting of the increments.

3.2.4. Isothermal Calorimetry

If Ca^{2+} or Mg^{2+}-binding leads to an enthalpy change (ΔH), either exothermic or endothermic, their affinity constants can be determined by isothermal calorimetry (ITC) using a MicroCal VP-ITC Microcalorimeter (Microcal Inc). This method, known since 1989 (46), has been applied for Ca^{2+}- and Mg^{2+}-binding experiments only recently and only in a few cases (37, 39, 47), but is expected to become a very important tool in the next future since it is fast (circa 2 h), nearly completely automated, and yields not only the affinity constants but also the enthalpy changes of each binding step. As for the true ΔH, it should be stressed that divalent cation binding to CaBPs implies exchange of protons and that these proteins in turn will be buffered by the working buffer leading to a positive or a negative heat release, depending on which type of proton buffer is used (see Note 4).

In practice circa 50 μM of the metal-free protein is titrated with 50 successive automatic injections of 5–10 μl each of a stock solution of 0.5–2 mM divalent cation. The additions were 4 min apart to allow heat accompanying each increment to return to the baseline prior to the next addition. For each addition the molar heat (Q_t) was measured as a function of the total ligand concentration (X_t):

$$Q_t = nP_t \Delta H \cdot V \left\{ \begin{array}{l} 1 + X_t / (nP_t) + 1 / (nK_a P_t) \\ -\left[(1 + X_t / (nP_t) + 1 / (nK_a P_t)^2 - 4X_t / (nP_t) \right]^{0.5} \end{array} \right\} / 2, \qquad (6)$$

where n is the number of sites, Pt is the total protein concentration, K_a is association constant, and V is the cell volume. The differential heat (dQt) is then fitted to various models by using a nonlinear least squares minimization method using Microcal Origin ITC software. The free energy of binding (ΔG) and entropy (ΔS) is obtained using the classical thermodynamic formulas $\Delta G = -RT \ln Ka$ and $\Delta G = \Delta H - T\Delta S$, where R is the gas constant and T is the absolute temperature in kelvin.

3.3. Mg^{2+} Binding

Under physiological ionic conditions at low Ca^{2+} concentrations many EF-hand-containing CaBPs interact with Mg^{2+} with affinities ranging from 10^2 to 10^5 M^{-1}. The binding of Mg^{2+} can be measured by equilibrium dialysis or by the fluorescent indicator method, but unfortunately not by flow dialysis since there is no stable radioactive isotope of Mg^{2+} with a long enough half-life time. A method well suited for Mg^{2+}-binding is equilibrium gel

filtration, first proposed by Hummel and Dryer (48). This method takes advantage of the fact that on a gel filtration column the protein is rapidly and efficiently brought to equilibrium with the buffer in which the gel matrix is equilibrated. In practice 0.5–1 ml of a protein solution is applied to a 0.8×40 cm column of Sephadex G-25 equilibrated in the working buffer plus a given concentration of free Mg^{2+}, and eluted at 0.4 ml/min. In the eluent fractions the protein concentration is monitored by its UV absorbance and Mg^{2+} by AAS. Analysis of three protein-containing fractions across the protein peak allows us to verify if the equilibrium is reached, which is the case when the bound Mg^{2+}-to-protein ratio is the same in the three fractions. In the method originally proposed the protein sample was loaded without any addition of the ligand. However, in Mg^{2+}-binding experiments it is preferable for kinetic reasons that the loaded protein already contains Mg^{2+}. A practical and rapid way of generating a Mg^{2+}-binding isotherm is to chromatograph first the CaBP in buffer containing 1 mM Mg^{2+} and, after analysis, rechromatograph each of the protein-containing fractions on columns equilibrated in lower Mg^{2+} concentrations (from 50 to 500 μM). Excess Mg^{2+} dissociates and a new equilibrium is rapidly reached. When performing Hummel–Dryer experiments in buffer containing less than 50 μM Mg^{2+}, the flow rate must be reduced to 0.1 ml/min. Since generating a correct isotherm takes 3–5 days, the determination of the Mg^{2+}-binding isotherms now constitutes the rate-limiting step in the unraveling of the ion-binding parameters of a given CaBP.

3.4. Analysis of the Binding Data

3.4.1. Stoichiometric, Microscopic, and Intrinsic Constants

The above methods yield model-independent isotherms which describe the binding of the ion to the protein. They provide macroscopic, also named stoichiometric binding constants, K_n, which refer to the binding of the first, second, etc. Ca^{2+} to the protein (49); for an excellent review see ref. (50). The most general description of the equilibria in terms of stoichiometric binding is provided by the Adair-Klotz model (51), which analyzes the binding data in terms of a set of constants that describe the binding of the first, second, … nth Ca^{2+} ion to the protein. The binding then obeys the following equation:

$$r = \frac{\left(K_1[Ca^{2+}] + 2K_1K_2[Ca^{2+}]^2 + \ldots nK_1K_2\ldots K_n[Ca^{2+}]^n\right)}{\left(1 + K_1[Ca^{2+}] + K_1K_2[Ca^{2+}]^2 + \ldots K_1K_2\ldots K_n[Ca^{2+}]^n\right)}, \quad (7)$$

where r is the ratio of bound Ca^{2+} per protein and n is the number of Ca^{2+}-binding sites. These stoichiometric constants yield information on the pace of the successive binding steps, but not on the affinity constant of any particular site. In a CaBP with four functional Ca^{2+}-binding sites I, II, III, and IV and the affinity constants

k_I, k_{II}, k_{III}, and k_{IV} (called microscopic or site-binding constants), the relationship between K_n and these constants is as follows (49):

$$\begin{aligned} K_1 &= k_I + k_{II} + k_{III} + k_{IV} \\ K_1K_2 &= k_Ik_{II} + k_Ik_{III} + k_Ik_{IV} + k_{II}k_{III} + k_{II}k_{IV} + k_{III}k_{IV} \\ K_1K_2K_3 &= k_Ik_{II}k_{III} + k_Ik_{II}k_{IV} + k_{II}k_{III}k_{IV} \\ K_1K_2K_3K_4 &= k_Ik_{II}k_{III}k_{IV}. \end{aligned} \tag{8}$$

It should be noticed that the microscopic k values may change during the binding process, e.g., the affinity of site II for Ca^{2+} may be different depending on the presence or not of Ca^{2+} in site I. Cornish-Bowden and Koshland (49) analyzed the binding isotherm in terms of the intrinsic affinity constant, K'_n, the meaning of which is intuitively the easiest understood since it represents the mean affinity constant of site n in a protein. If all the microscopic constants are identical there is only one intrinsic constant and the relation between stoichiometric and intrinsic constant is as follows: $K_1 = 4\,K'$, $K_2 = 3/2\,K'$, $K_3 = 2/3\,K'$, and $K_4 = ¼\,K'$. If the k values do not change during the titration (i.e., in a noncooperative system), but are different, the four different intrinsic constants, K'_1, K'_2, K'_3, and K'_4, describe the binding isotherm. In many instances, as in the first part of this chapter, the binding parameters are expressed as dissociation constants K_d, since these are closest to the notion of the Ca^{2+} concentration which induces half maximal binding or response: in most (simplified) cases $K_d = 1/K'$.

In the case of positive cooperativity in a two-site system the relationship between the intrinsic constants is as follows: $K'_1 < K'_2$ and the degree of allostery is given either by the ratio $k_{I,II}/k_I$ or by $\Delta G = \Delta G_{I,II} - \Delta G_I$, i.e., by the difference between the change in free energy when Ca^{2+} binds to a given site in the presence ($\Delta G_{I,II}$) or absence (ΔG_I) of bound Ca^{2+} in the second site. However a much more popular cooperativity index is given by the empirical Hill model, with

$$r = nK^{nH}[Ca^{2+}]/(1 + K^{nH}[Ca^{2+}]), \tag{9}$$

where n is the number of sites, n_H the Hill coefficient, and K an apparent binding constant, not related to the stoichiometric, intrinsic, or macroscopic constants (49). Linearization of Eq. 9 yields the widely used Hill function:

$$\log\{r/(n-r)\} = \log K + n_H \log[Ca^{2+}]. \tag{10}$$

3.4.2. The Ca^{2+}/Mg^{2+} Antagonism

In most cases the affinities of CaBPs for Ca^{2+} are modulated by millimolar concentrations of Mg^{2+}, either by direct competition at the so-called Ca^{2+}/Mg^{2+} mixed sites or by indirect antagonism in proteins with Ca^{2+}-specific sites. A primordial task after the

Ca^{2+}-binding isotherms have been collected at different Mg^{2+} concentrations is to determine which model of antagonism is functioning.

1. In the model of *direct competition* the EF-hand site binds either Ca^{2+} or Mg^{2+} and the shift of the isotherms to higher $[Ca^{2+}]$ is unlimited. The competition model obeys the following equation:

$$K_{Ca} / K_{Ca.app} = 1 + K_{Mg}[Mg^{2+}], \tag{11}$$

where K_{Ca} and $K_{Ca.app}$ are the Ca^{2+}-binding constants in the absence and presence of Mg^{2+}, respectively, and K_{Mg} the Mg^{2+}-binding constant in the absence of Ca^{2+}. Examples of the analysis of simple competition models have been presented for troponin C (52) and parvalbumin (53); complex, but still direct, competition models have been described for sarcoplasmic Ca^{2+}-binding proteins (3, 54, 55).

2. *Indirect Ca^{2+}–Mg^{2+} antagonism* is exemplified by calmodulin (4). In this protein Mg^{2+} does not bind to the same sites as Ca^{2+}, but to auxiliary sites (56), physically different from the four EF-hands. Calmodulin can bind 4 Ca^{2+} and 4 Mg^{2+} simultaneously. The Ca^{2+}- and Mg^{2+}-binding sites influence each other's affinity due to negative free energy coupling between the sites. The amount of bound ligand per mole of site is given by (see ref. 4)

$$\text{Bound } Ca^{2+} / \text{site} = \frac{1 + (K_{Mg(Ca)}[Mg^{2+}])^{-1}}{1 + (K_{Ca(Mg)}[Ca^{2+}])^{-1} + (1 + K_{Ca}[Ca^{2+}])^{-1}(K_{Mg(Ca)}[Mg^{2+}])^{-1}}; \tag{12}$$

similarly:

$$\text{Bound } Mg^{2+} / \text{site} = \frac{1 + (K_{Ca(Mg)}[Ca^{2+}])^{-1}}{1 + (K_{Mg(Ca)}[Mg^{2+}])^{-1} + (1 + K_{Mg}[Mg^{2+}])^{-1}(K_{Ca(Mg)}[Ca^{2+}])^{-1}},$$

where K_{Ca} and K_{Mg} are the association constants for Ca^{2+} and Mg^{2+}, respectively, in the absence of the other ligand, and $K_{Ca(Mg)}$ and $K_{Mg(Ca)}$ the corresponding constants in the presence of infinite concentrations of the second ligand. As in the model of straight competition, upon increasing the Mg^{2+} concentrations the Ca^{2+}-binding isotherms shift to higher free Ca^{2+} concentrations, but in this model the shift is not unlimited and levels off at the isotherm dictated by $K_{Ca(Mg)}$.

3.4.3. Conclusion

The examples mentioned above show the great diversity of the thermodynamic behavior of different CaBP families, diversity necessary to cope with the complexity of the Ca^{2+} signal. The kinetics of Ca^{2+} and Mg^{2+}-binding, not treated here and in general rarely studied, adds a new dimension to the action of CaBPs in the cell.

4. Notes

1. Occasionally, we used flow dialysis cells with a spiraled (Feldman cell) lower compartment instead of the cubic lower chamber. Its smaller volume assures a faster installation of a steady state between the two compartments.
2. In our studies the working buffer for nearly all methods is 50 mM Tris–HCl, pH 7.5 150 mM KCl, except for ITC experiments where a Mops buffer, pH 7.4, 100 mM NaCl is used (its protonation enthalpy is negligible).
3. To a solution (500 µl) containing 15 µM of protein and 1 µM fluorescent Ca^{2+} indicator (BTC or Mag-fura-2) 20 µM increments of Ca^{2+} were added and the fluorescence excitation spectra were monitored after each addition. For BTC the fluorescence intensities at 401 (increasing) and 469 nm (decreasing) were ratioed with respect to that at the isosbestic point at 433 nm to eliminate interferences or lamp intensity changes. For example, for Mag-fura-2 the data were extracted at 332 (increasing), 352 (isosbestic point) and 385 nm (decreasing). The signal changes were normalized from 0 (nominaly zero [Ca^{2+}]) to +1 (saturating [Ca^{2+}]), yielding ΔS at the respective wavelengths which were averaged to yield ΔS_i. The concentration of free Ca^{2+} was then calculated by extrapolation of these ΔS_i values at each increment to the Ca^{2+}-binding isotherms of BTC or Mag-fura-2. The latter were estimated independently and yielded affinity constants of 1.4×10^5 and 1.8×10^4 M^{-1} for BTC and Mag-fura-2, respectively. Protein-bound Ca^{2+} was then calculated by subtracting the free [Ca^{2+}] from the total amount of added Ca^{2+} *plus* the initial contamination determined by atomic absorption.
4. The heat production or absorption (Q) as a function of the degree of saturation of a site (α) is given by $Q = \alpha(\Delta H_{Ca} + n\Delta H_{proton})$, where ΔH_{Ca} is the enthalpy of the Ca^{2+}-binding process and n and ΔH_{proton} are the number of protons liberated and the protonation enthalpy of a given buffer, respectively. When the same reaction is studied in two buffers of different protonation enthalpy, ΔH_{p1} and ΔH_{p2}, the value of n can be formulated by $n = (Q_1 - Q_2)/[\alpha \times (\Delta H_{p1} - \Delta H_{p2})]$. Thus, by calorimetric monitoring of a reaction in two different buffers one can calculate the proton release and determine subsequently the real ΔH_{Ca} of the process.
5. The Ca^{2+}-dependent exposure of a hydrophobic patch on the surface of a CaBP I is measured by fluorescent indicators such as 8-naphtalene-1-sulfonate (ANS), 2-p-toluidinyl-naphtalene-6-sulfonate (TNS), and 4,4′-dianilino-1,1′-binaphtyl-

5,5′-disulphonate (BISANS). In our studies nearly always TNS (Sigma) was used: a 400 µl solution of 40 mM TNS and 1 µM protein was excited at 322 nm and the emission spectra measured from 380 to 540 nm.

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