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CALCIUM-BINDING PROTEINS

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I. Introduction

Calcium is of widespread and fundamental importance in biochemistry, for the calcium ion functions as a second messenger, that is, one whose signals are propagated by proteins specifically evolved for this purpose. It is regarded as one of the most important bioinorganic elements (*1*). Apart from its relevance to biological solid-state materials, Ca^{2+} has key roles in many physiological processes. The Ca^{2+} ion has a radius of 100 pm and can be compared to Mg^{2+} (72 pm) with its

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strict coordination requirements, having coordination numbers from 6 to 8. In its biological coordination chemistry it has a preference for O-donor ligands from aminoacid side-chain carboxylate or hydroxyl groups, peptide carbonyl oxygens, or H_2O. The hexaaqua ion is very labile and has an H_2O exchange "off" rate constant of $5 \cdot 10^8$ s^{-1} (*2*). This could in a simplistic way be thought of as an upper limit for the "on" rate of Ca^{2+} to its binding site. Higher on rates are possible, however, when outer-sphere complexes are favorable and especially when the binding site is highly negatively charged as discussed in (*3*, *4*).

A review on calcium-binding proteins is a quite difficult task. During work on this review we have found that there are now an immense number of such proteins, making some selection necessary. Even for those proteins selected we have no claims of completeness in our coverage. We have therefore divided the proteins (or more correctly protein domains), into those that are (a) intracellular, (b) calcium mediated membrane bound, and (c) extracellular. The intracellular calcium-binding proteins are dominated by the EF-hand family (defined later). This can be clearly seen from the recent book "Guidebook to the Calcium Binding Proteins" (*5*), in which the EF-hand proteins occupy 180 of a total of 230 pages. Likewise, in the section on intracellular proteins in this review we have chosen to concentrate on the EF-hand family.

In the section on calcium-mediated membrane-binding proteins we have chosen to be more varied and discuss proteins interacting with membranes from the inside as well as from the outside. In this section it might be more proper to talk about protein domains than proteins. In general it is the location of the calcium-binding domain that has been used to define whether it is intra- or extracellular or membrane bound. For example, the cadherins are treated as extracellular in this review even though they are truly membrane proteins, and the various domains from the blood coagulation factors are treated as membrane binding (Gla domains) as well as extracellular (EGF and serine protease domains). We hope this will not be too confusing for those used to other definitions; however, in this review focused on the calcium ion we found this division natural.

We are aware that several important calcium-binding proteins have been neglected and that our selection of proteins is clearly biased by our own particular interests. We have also included some odd proteins such as conantokins and neglected more important ones such as phospholipase A_2.

II. Intracellular EF-Hand Calcium-Binding Proteins

A. Introduction

The EF hand is by far the most common motif for intracellular calcium-binding proteins. Briefly, it is an approximately 30 amino acid–long peptide chain composed of a central calcium-binding loop flanked by two alpha helixes. The name EF-hand was coined by Kretsinger (*6*) and refers to the resemblance of the E and F helices of parvalbumin to a hand with the thumb and index finger extended. The calcium ion is bound by oxygen ligands contributed mostly from carboxylic acid side chains from aspartic or glutamic acid. Often one ligand is contributed by water, and in rare cases, as discussed later, the backbone carbonyl oxygens can contribute ligands. The sequence of a typical EF-hand is shown in Fig. 1 and a stick figure of its calcium-binding loop is shown in Fig. 2. The ligating residues are relatively highly conserved and have been used to identify almost a thousand EF-hands in over 150 proteins (*7*). However, of those identified, approximately a third are believed to have lost their ability to bind calcium tightly, that is to say, with a dissociation constant of 10^{-5} to 10^{-8} M as is typical for these proteins. As expected, those that have

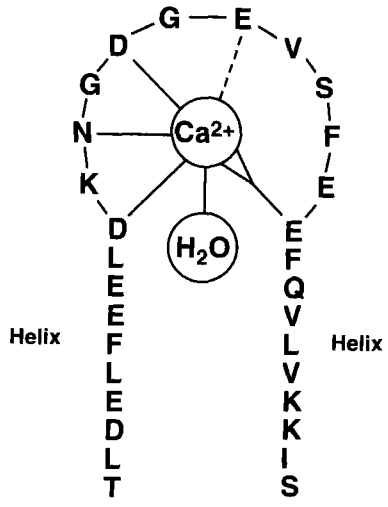


FIG. 1. A schematic drawing of the second EF-hand calcium-binding subdomain from calbindin D_{9k} (residues 45–74). Ligands from side-chain carboxyls are indicated in solid lines; the ligand from the backbone carbonyl is indicated by a dashed line.

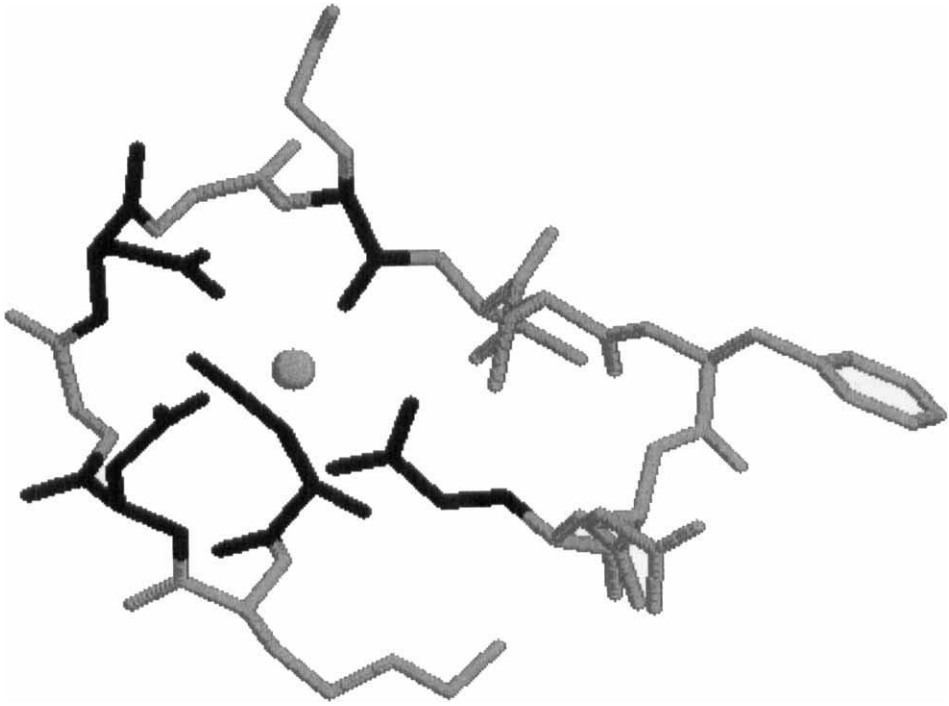


FIG. 2. A stick figure of the second EF-hand binding loop from calbindin D_{9k} (residues 54–65). The carboxylic side chains and backbone carbonyls that ligand the calcium ion are indicated in black.

lost the ability to bind calcium are also those whose sequences vary the most from the canonical EF-hand sequence.

A second feature that is conserved among the EF-hand proteins is the arrangement of hydrophobic residues on the interior faces of the two helices and the central loop. These residues are clustered as a hydrophobic surface and help to explain another key feature of EF-hand motifs. This feature is that except on rare occasions, EF-hands occur in pairs with their hydrophobic surfaces joined together and the calcium loops paired in a small β-sheet interaction. The two EF-hands in the pair share a pseudo-axis of rotation centered between the four helices. This four-helix bundle arrangement is very stable and is observed even in non-calcium-binding proteins such as hemerythrin (*8*) and cytochrome B_{562} (*9*). For example, the stability of calbindin D_{9k}, a small calcium-binding protein composed of a single EF-hand pair, in its calcium-loaded state is so high that it cannot be denatured by urea or heating to 100°C (*10*).

Although EF-hands tend to occur in pairs, proteins can contain anywhere from 1 to 8 EF-hand motifs with the most common number being 4. A survey of a large number of known EF-hand calcium-binding proteins has been presented by Nakayama, et al. (*7*). The functions of EF-hand proteins can be divided into two categories, signaling and buffering, and the distinction between the two groups is determined by their structural response to calcium binding. In the case of signaling proteins, the binding of calcium induces a conformational change, which renders the protein in its active form. Once active, the protein is able to bind a target protein or peptide to carry out its function. The classical example of this type is calmodulin. The activation process is tightly regulated by the concentration of calcium in the cell, which is 10^{-8} M in a resting cell but can increase by several orders of magnitude as a result of calcium intake from outside the cell or release from internal stores.

In the case of the buffering proteins, calcium binding has little or no significant effect on the structure of the protein and the function of the protein is most likely limited to its calcium-binding role. The best studied example of the latter is calbindin D_{9k}.

The majority of the EF-hand family of calcium-binding proteins occur within the cell. However, there are several recent cases in which EF-hand motifs have been identified in proteins expressed outside of the cell or on the cell surface. These are discussed in Sections III and IV. In this section we will concentrate our discussions on intracellular calcium-binding proteins and give some examples of the best-known and most studied examples of such proteins. This is by no means an exhaustive list of EF-hand proteins, only a consideration of some examples that have been extensively studied from a structural perspective. Excellent introductions to the entire EF-hand family by Kawasaki and Kretsingar (*11*) and Celio (*5*) are recommended to those interested in other members of this fascinating family of proteins.

B. Calmodulin

That the EF-hand proteins are often referred to as the calmodulin superfamily of calcium-binding proteins is no coincidence. Though the term "EF-hand" was coined for parvalbumin (*6*), calmodulin is the most well characterized of the EF-hand proteins with respect to every aspect from molecular structure to physiological role. It serves a central role as a calcium-sensitive second messenger and is found in all eukaryotic organisms (*12*). Rather than having a single target as is

common for signaling proteins, calmodulin has over 100 targets and thus regulates a wide range of cellular function including signal transduction, DNA synthesis, secretion, motility and cell division (*11*, *12*). It is composed of a single chain of 148 amino acids with a molecular mass of approximately 18 kDa. It contains four EF-hand calcium-binding subdomains that are arranged pairwise in two domains separated by a central linker. All four sites are active and bind calcium with high affinity, with the carboxy-terminal EF-hand pair having slightly higher affinity ($K_D = 10^{-6}$ M) than the amino-terminal pair ($K_D = 10^{-5}$ M). The first structure of calcium-loaded calmodulin from rat was determined by X-ray crystallography in 1985 (*13*) and was later refined to high resolution in 1988 (*14*).

These structures reveal a surprising architecture for the protein in that the two EF-hand pair domains are separated by a long, apparently rigid helix (Fig. 3). This "dumbbell" shape of calmodulin was surprising because biochemical data indicated that the target binding site was evenly distributed between the two domains, but its target peptides were obviously too small to span the distance between the two domains. In addition, small-angle X-ray scattering and neutron scattering experiments indicated that the solution form of calmodulin was much more compact than that determined by X-ray crystallography (*15*, *16*). Further X-ray structures of calmodulin from human (*17*), *Drosophila* (*18*) and *Paramecium* (*19*) spp. also demonstrated the presence of a central helix.

It was not until a high-resolution structure of calmodulin in solution was obtained by nuclear magnetic resonance (NMR) spectroscopy that the apparent paradox of calmodulin's structure was resolved. Al-

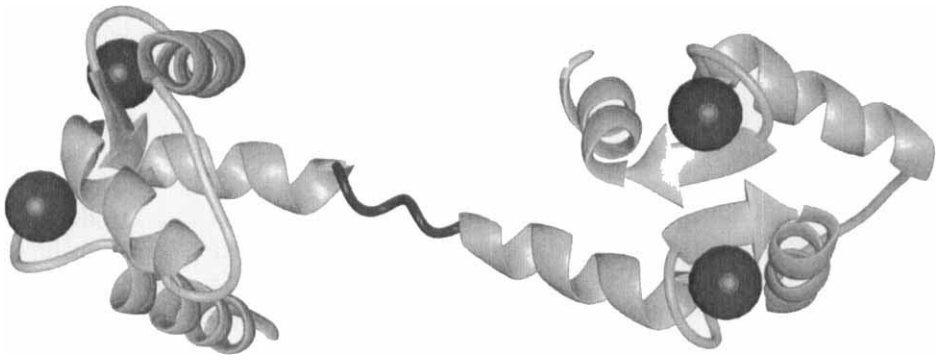


FIG. 3. A ribbon diagram of active $(Ca^{2+})_4$ human calmodulin (*17*). The central flexible linker separating the two EF-hand pair domains is indicated in dark gray.

though there was good agreement between the structures determined by X-ray crystallography and NMR spectroscopy, the central linker was found to be nonhelical in solution (*20*). Furthermore, measurements of the dynamics of the backbone using NMR relaxation data showed that this linker was more flexible than the surrounding helices (*21*).

Target binding by calmodulin is an interesting case of molecular recognition. Although calmodulin binds a large number of target proteins, it binds with high affinity, often in the nanomolar range. Therefore, one might have expected that they share a common calmodulin binding sequence. However, the many calmodulin recognition sequences share little sequence homology and only resemble one another in the general properties of being 14 to 26 amino acids long, hydrophobic, and basic (*22*).

Both NMR spectroscopy and X-ray crystallography played key roles in understanding the target binding of calmodulin. In this case, the two methods show much more agreement. Three high-resolution structures of complexes between calmodulin and a target protein have been published, two by X-ray crystallography on complexes with peptides with the smooth muscle light-chain kinase (smMLCK) (*23*) and calmodulin-dependent protein kinase II (CaMKII) (*24*), and one by NMR spectroscopy on the complex with a peptide from skeletal muscle light-chain kinase (skMLCK) (*25*). This latter structure is shown in Fig. 4.

The smMLCK and skMLCK complex structures resemble one another, as is expected because the peptides share a degree of sequence homology. The main features of the complex are two hydrophobic anchors at opposite ends of the peptide that interact with hydrophobic residues in the calmodulin-binding pocket. In addition, electrostatic interactions between the basic residues of the peptide with glutamic acid residues in both domains of calmodulin are observed. In the case of CaMKII, the hydrophobic anchors are present, but are closer to one another in the peptide sequence. Calmodulin adapts its binding to this sequence in two ways. First, because the two domains are connected by a flexible tether, they are able to rearrange with respect to one another and the target peptide so as best to accommodate the target peptide's hydrophobic amino acid pattern. The second adaptation occurs within the two binding clefts themselves. These clefts contain a larger than usual proportion of methionine residues that have a long side chain, thus giving them more degrees of freedom to adjust their conformations as necessary to accommodate the target peptide.

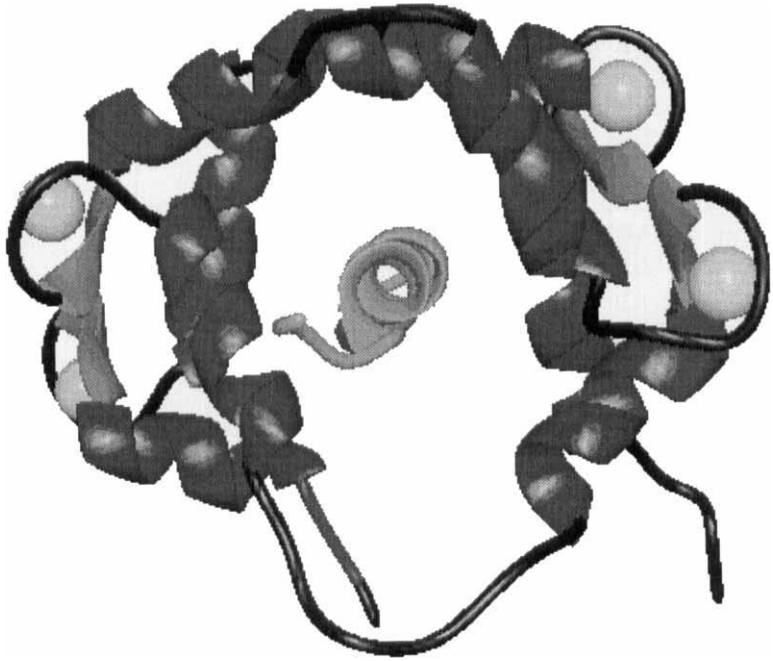


FIG. 4. A ribbon diagram of the binding of skMLCK peptide to calmodulin (*25*). The peptide and calcium ions are indicated in light gray and calmodulin in dark gray.

Although the structures of calmodulin bound to target peptides have yielded much information about the complexes and their plasticity, the mechanism by which binding of calmodulin to the target *proteins* activates them remains to be definitively demonstrated. This will require the structure of a calmodulin–protein complex. However, much evidence available now allows us to infer the mechanism of calmodulin-dependent activation. The strongest hypothesis is the pseudosubstrate or autoinhibition hypothesis, which was first suggested by Kemp *et al.*, who noted the similarity of the substrate and autoinhibitory region of smMLCK (*26*). Subsequently, it was demonstrated that removal of the autoinhibitory region by proteolysis could activate smMLCK in the absence of calmodulin (*27*). A recent structure of the autoinhibited form of calmodulin-dependent protein kinase I (CaMKI) clearly shows that its autoinhibitory region lies in the substrate binding site and that part of the calmodulin binding segment protrudes out, thus potentially able to bind calmodulin (*28*).

Though the vast majority of target proteins bind to calcium-activated calmodulin, there are cases in which calmodulin has been

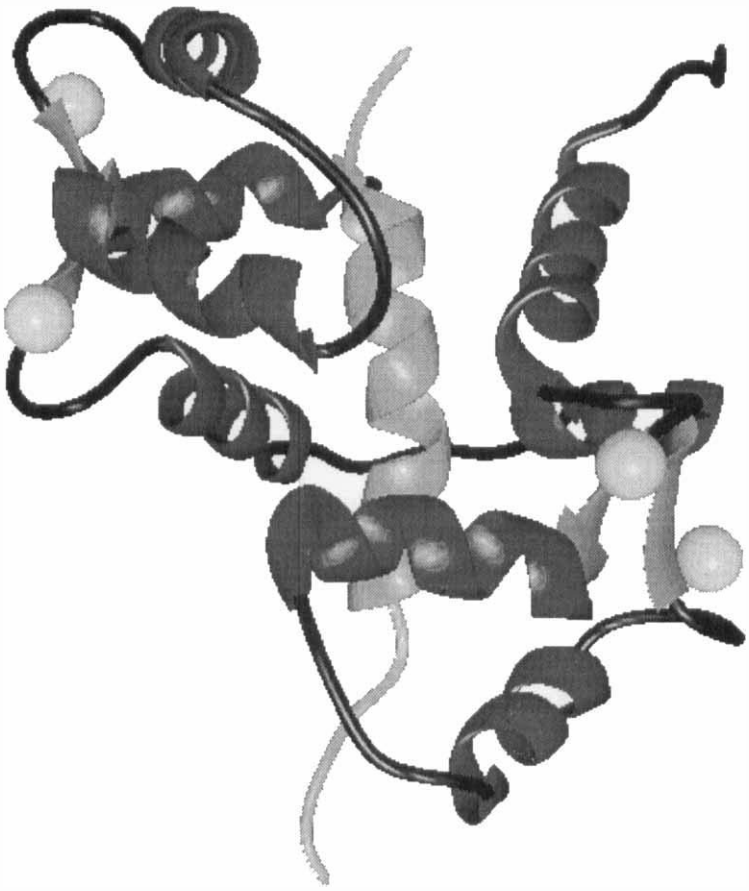


FIG. 4. (*continued*)

reported to bind proteins in the absence of calcium. However, in most cases they are believed to be affected by changes in calcium concentration. One example is that of neuromodulin, which binds calmodulin tighter in the absence of calcium at low ionic strength. The physiological significance of this binding is unclear because it is much weaker at physiological salt concentrations. Other evidence suggests that neuromodulin may act to bind and sequester calmodulin at the plasma membrane in a calcium-dependent manner (*29*, *30*).

The mechanism of calcium-induced activation of calmodulin has been and continues to be a debated topic. Since the first X-ray crystal structure of calcium-loaded calmodulin (*13*), models have been proposed for the structure of the apo form of calmodulin (*31*) based on

the structure of related troponin C, which was first crystallized with only two of four calcium-binding sites loaded with calcium (*32*, *33*). Briefly, the model predicted that the hydrophobic binding sites of calcium–calmodulin are closed and sequestered from solvent in the apo form. The general features of this model were confirmed ten years after the crystal structure of the calcium-loaded from by several solution structures of apo-calmodulin determined by NMR (*34–36*). The conformational change observed for the carboxy-terminal domain is shown in Fig. 5. Additionally, this structure demonstrated the autonomy of the two domains with respect to their conformational response to calcium binding being independent of the linker connecting the two domains (*37*).

C. Troponin C

Troponin C (TnC) is a calcium-binding protein that is part of the troponin complex localized on the thin filament of muscle fibers. The other two components of the complex are troponin I (TnI) and troponin T (TnT), which together with TnC are arranged in a heterotrimer. Two isoforms are known, the fast skeletal muscle (sTnC) and the cardiac and slow skeletal muscle (cTnC) forms. The structure of TnC is similar to that of calmodulin, with four EF hands organized pairwise in two domains. Indeed, our understanding of these two proteins has evolved in parallel, with a good deal of what has been learned about troponin C being applicable to calmodulin and vice versa. Like cal-

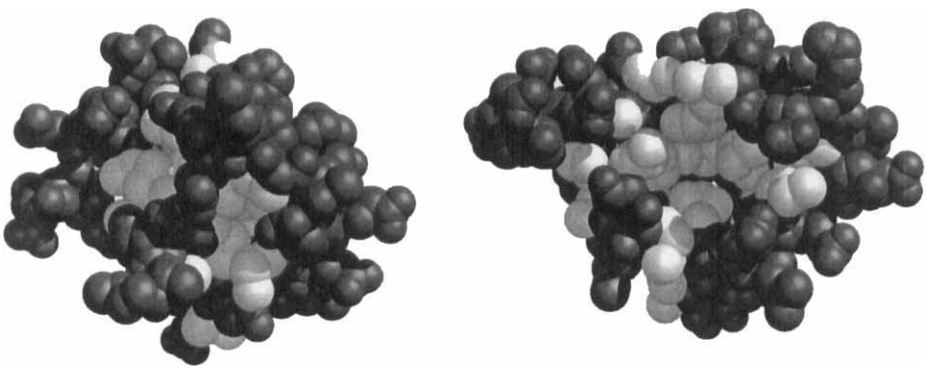


FIG. 5. A space-filling drawing of the conformational change in the TR_2C domain of calmodulin (*37*). The hydrophobic side chains lining the half of the target binding site in the TR_2C domain are indicated in white with the backbone and remainder of the side chains in gray.

modulin, all four calcium-binding sites in sTnC are active, with the carboxy-terminal sites being tighter than the N-terminal pair ($K_D \sim 10^{-8}$ M vs. $K_D \sim 10^{-6}$ M). However, in cTnC the first site is inactive. Unlike calmodulin, which binds to a large array of target proteins, TnC has one very specific function, namely the cyclic binding and release of TnI in a calcium-dependent manner, which in turn regulates muscle contraction.

The structure of sTnC was first determined by X-ray crystallography and remarkably in a half-saturated $(Ca^{2+})_2$ state (*32*, *33*). These initial structures formed the basis for modeling of the mechanism of calcium-induced activity of troponin C (*38*) as well as the calcium-induced activation of calmodulin (*31*). Studies of the solution structures of sTnC added the structures of the $(Ca^{2+})_4$ calcium-saturated form (*39*) and the apo and calcium-saturated forms of the N-terminal, regulatory, domain (*40*, *41*) to the already known half-saturated form. Together, these paint a complete picture of the structural changes induced by calcium binding on troponin C and have led to a model for troponin C's role in muscle contraction. The model predicts that in the resting state, only the C-terminal domain of sTnC is calcium loaded and bound to TnI at its N-terminal domain. Actin is also bound to TnI in an inhibitory complex. Upon binding of calcium to TnC's N-terminal domain, a hydrophobic patch is opened and binds to the inhibitory and C-terminal domains of TnI, thus releasing actin from TnI. The actin is thus available for binding to myosin, which leads to the muscle contraction. Support for another part of this model was provided by studies of the interaction of sTnC and its binding target domain on TnI by NMR (*42*), which confirmed that TnI interacts with the regulatory domain of TnC. Further details of the mechanism of action of troponin C have also been provided by solution structural studies, including mutants of troponin C aimed at dissecting the role of individual residues in the process. The final bidentate glutamate ligand in the calcium-binding loops has been an especially attractive target in troponin C (*43*, *44*) as well as calmodulin (*45*, *46*). These studies have demonstrated these to be key residues in the calcium-induced activation process. Whether they are the only keys or part of an interplay between a number of calcium–ligand and other interactions is a subject of continuing study.

D. S100 Proteins

The S100 proteins make up a distinct subfamily within the EF-hand family. They share structural characteristics of being small

(9–12 kDa), being acidic, containing only 2 EF-hands, and having a homology of between 25 and 40%. They also share one feature that is unique among calcium-binding EF-hand proteins, that is, the insertion of two residues in the first EF-hand, which alters the conformation of the calcium-binding loop such that the ligands are contributed from the peptide backbone carbonyl oxygens instead of the side-chain carboxyl groups as in a standard EF-hand. This variation is known as a "pseudo-EF-hand." Beyond this, they differ widely with respect to expression, activity, oligomer state, and conformational response to calcium binding.

1. Calbindin D_{9k}

Calbindin D_{9k}, also known as ICBP and S100D, is expressed highly in the intestine, placenta, and uterus, and has been suggested to be involved in calcium transport, though a definitive demonstration of its function remains to be accomplished. It has been shown to interact with the calmodulin-binding domain of the plasma membrane calcium pump (*47*), though it is unclear if this interaction plays a significant functional role. It is a unique member of the S100 family in that it is monomeric whereas most members of this subfamily are homo- or heterodimeric. From a structural perspective, calbindin D_{9k} is the most extensively studied of the S100 subfamily. It is precisely its small size as well as its stability that makes it an attractive target for structural studies. The first X-ray structure of calcium-loaded calbindin D_{9k} was determined in 1981 (*48*) and was later refined to 2.3 Å (*49*), and then to 1.6 Å (*50*). Solution structures have also been determined for several forms of bovine calbindin D_{9k} including $(Ca^{2+})_2$ (*51*), apo (*52*, *53*), $(Cd^{2+})_1$ (*54*) and $(Cd^{2+})_2$. In addition, the structure of the porcine protein has been determined (*55*) by NMR methods.

These studies when taken together show another unique feature of calbindin D_{9k}: the relatively small effect that calcium has on the conformation of the protein. On going from the apo to calcium-loaded state, only a minor adjustment of the secondary structure is observed, and this is limited to the second EF hand. The structure of the singly loaded $(Cd^{2+})_1$ state showed that most of these changes occur upon binding of the first ion, which binds in the second, C-terminal binding site (*54*). This lack of a large structural response to calcium binding, which is typical for the calcium sensors such as calmodulin and troponin C, indicates that calbindin D_{9k}'s function is probably not one of signaling but more likely one of regulating calcium homeostasis.

Although ion binding has little effect on the structure of calbindin, it has been shown to have a large effect on the dynamics and stability of the protein. Calbindin D_{9k} has served as a convenient model system for studies of protein dynamics by NMR and other techniques. Results from studies of hydrogen–deuterium exchange (*56*, *57*) have shown that the structure of calbindin can be significantly stabilized by ion binding. The effect is greatest in the binding loops, but it is also propagated through hydrogen-bonding networks in the helices. Similar studies on the fast (nano- to picosecond) time-scale dynamics by spin-relaxation techniques (*58*, *59*) have shown that ion binding exerts an effect here as well but is localized to the C-terminal binding loop, which is more dynamic in the apo than in the calcium-loaded form.

Structural studies of calbindin D_{9k} have also yielded several unique observations of a more general nature. The first was the observation that the purified protein appeared heterogeneous in isoelectric-focusing experiments. This heterogeneity was traced to an isoaspartyl linkage formed by deamidation of an Asn-Gly pair (*60*). A second apparent heterogeneity in the protein preparation was identified as well. However, instead of being a configurational heterogeneity it was found to be a conformational heterogeneity traced to the *cis–trans* isomerization of a proline in the linker between the two EF hands (*61*). This result, later confirmed by crystallographic studies (*50*), was one of the first direct demonstrations that such isomers of proline occur at equilibrium.

Calbindin D_{9k} has also proved to be a convenient model system for studies of calcium binding and cooperativity (*62*). Because it has only two calcium ions, it presents a simpler system than those proteins that have four or more calcium binding sites.

2. *S100β*

S100β occurs most commonly as S100B, a $\beta\beta$ dimer, but can form heterodimers ($\alpha\beta$). It is one of the better characterized members of the S100 family. It was the first protein given the designation S100, which was given to S100β when it was first isolated and means, simply, that it is partially soluble in 100% saturated ammonium sulfate (*63*). Thus, though the name S100 does not indicate any functional aspect, it has since been used by some to designate this entire family of proteins due to their homology to S100β and common chromosomal localization (*64*).

The exact function of S100β is not known. However, it has been implicated in a number of cellular functions such as cell growth,

apoptosis, and energy metabolism. It is most abundant in the nervous system, especially the glial cells, but it can also be found in other tissues such as the skin, testis and cartilage (*65*). It has also been shown to exhibit extracellular functions related to neuronal and glial cell growth and proliferation (*66*). S100β contains two cysteine residues (68 and 84), and the S100B dimer is thus able to form a covalently linked dimer. Although the disulfide linkage is not necessary for dimerization, it has been shown to be essential for its extracellular function and some intracellular functions (*67*).

Calcium binding causes a conformational change in S100B, which produces an increase in hydrophobic surface area, similar to calmodulin and troponin C. However, the details of this conformational change are only now beginning to come to light. The high-resolution structure of S100B was first determined in apo form for both the bovine (*68*) and rat (*69*) proteins. The rat S100B structure is shown in Fig. 6. These structures showed that the protein is composed of a dimer of two regular EF-hand pairs as expected. The dimer interface is an X-type four-helix bundle made up of helices 1, 4, 1′, and 4′. However, the structures differed significantly from one another in several important respects. First, the position of helix 3 in the second EF hand in each monomer differs enormously. Second, a small extra helix in the linker between helices 2 and 3 is reported for the bovine structure but not observed in the rat structure. Some suggest that these differences may be due to modeling rather than actual difference in the structures of the proteins from different species (*70*), but independent evidence will be required to resolve this question satisfactorily.

Several structures of the calcium-bound form of S100B determined by X-ray crystallography of the bovine protein (*71*), and by NMR spectroscopy of the rat (*72*) and human (*73*) proteins, have been recently determined. The structures confirm several features predicted earlier (*69*, *70*), namely that the major conformational changes are in the linker between the two EF hands, helix 3, and the second calcium-binding loop. The dimer interface is essentially unchanged.

Evidence has also been recently obtained by NMR chemical shift analysis on the interaction of calcium-activated S100B and target proteins CapZ (*74*) and p53 (*75*). Here again, there are differences in the reported binding sites on S100B, which may be due to differences in the binding peptides.

3. *Calcyclin*

Calcyclin, also called S100A6 (*64*) or CaBP (*76*), is a dimeric protein similar to S100β in many respects. It is found in various tissues in-

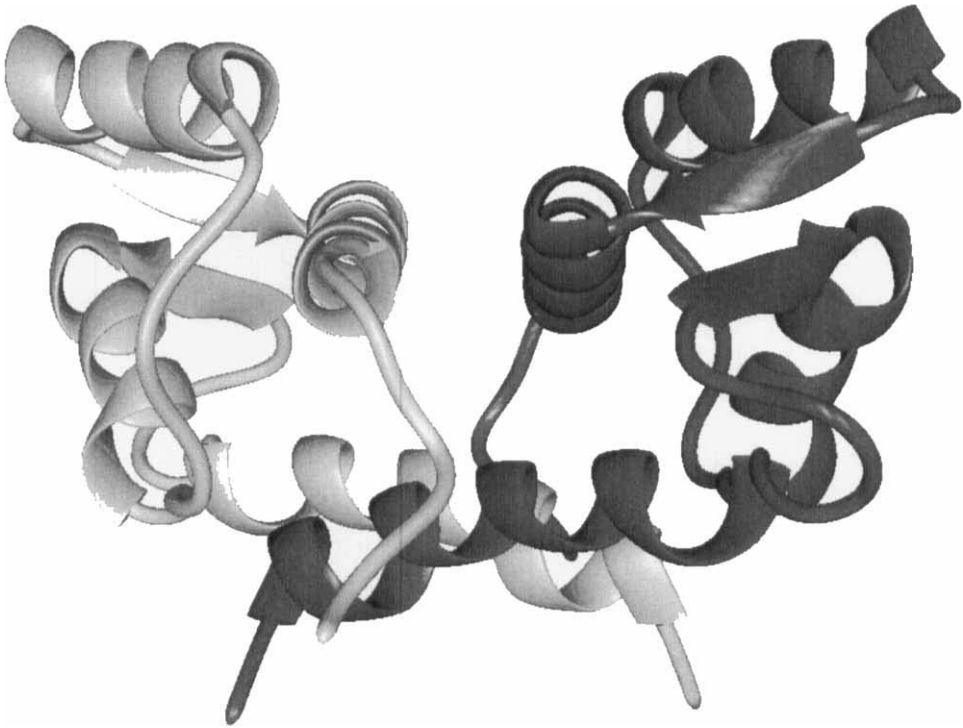


FIG. 6. A ribbon diagram of the structure of S100B as determined by Drohat *et al.* (*69*). The two members of the dimer are shaded differently to distinguish them. Helixes 3 and 3′, those believed to be involved in conformational change and target binding, are located at the top. Helixes 4 and 4′ lie at the dimer interface perpendicular to the plane of the figure; helixes 1 and 1′ lie under these parallel to the plane of the figure.

cluding lung, heart, platelets, and muscle. It undergoes a conformational change upon calcium binding and exposes a hydrophobic patch, which is most probably the target binding site. As was the case for calmodulin and troponin C, the structural characterizations of calcyclin and S100B have been closely linked. A solution structure of apo-calcyclin was determined by NMR spectroscopy (*77*), and the protein was found to be a symmetrical dimer whose interface was composed of helices 1, 4, 1′, and 4′, as was later found for S100B. However, there are differences between calcyclin and the two reported apo-S100B structures (*68*, *69*), especially in the region of helix 3. These differences may be genuine, but helix 3 is the least well determined segment of the apo-calcyclin structure, and a higher-resolution structure will be needed to settle the question of how different they really are. Characterization of the calcium-loaded form of calcyclin

has also been done by NMR methods (*78*). As opposed to S100B, only subtle changes in the conformation of calcyclin are observed upon calcium binding, and these are similar to those observed in calbindin D_{9k}. This result is surprising and in apparent conflict with calcyclin's role as a calcium sensor and data that indicate that a hydrophobic surface is opened upon calcium binding (*76*, *79*, *80*). The functional role and interactions with target proteins remain an area of intense study. One target protein has been identified and is expressed predominantly in the brain, similar to calcyclin itself (*81*, *82*). This suggests that calcyclin may act as a calcium-dependent neuronal signaling protein.

III. Calcium-Mediated Membrane-Binding Proteins

A. Introduction

Calcium-mediated membrane binding of proteins occurs both inside cells and outside. At first one might think that these events should be very different in these two different environments, with calcium concentrations in μM and mM, respectively. It is, however, not necessarily so.

Two extreme models for the calcium-mediated membrane binding may be envisaged. The first one, exemplified by recoverin, is when calcium binding to the protein causes a structural change that, somewhere else, exposes a hydrophobic patch that may bind to the membrane without any direct involvement of the calcium ion. In the case of recoverin the hydrophobic patch is in fact a myristoyl chain attached to the N terminus of the protein. The other extreme case would be where the calcium ions form a link between the protein and membrane surface. This has for some time been a working model for the binding of Gla-rich domains from blood coagulation proteins to phosphatidyl serine–containing membranes. More recently, however, another, more specific, model for this interaction has emerged, as will be discussed later. In our opinion the mode with the calcium ion as a glue between two negatively charged surfaces will probably not be found because there will be no specificity in such an interaction. However, there are certainly examples in which the calcium ion is coordinated to a protein as well as to a phospholipid head group. We will now discuss a few cases of calcium-mediated protein binding to membranes.

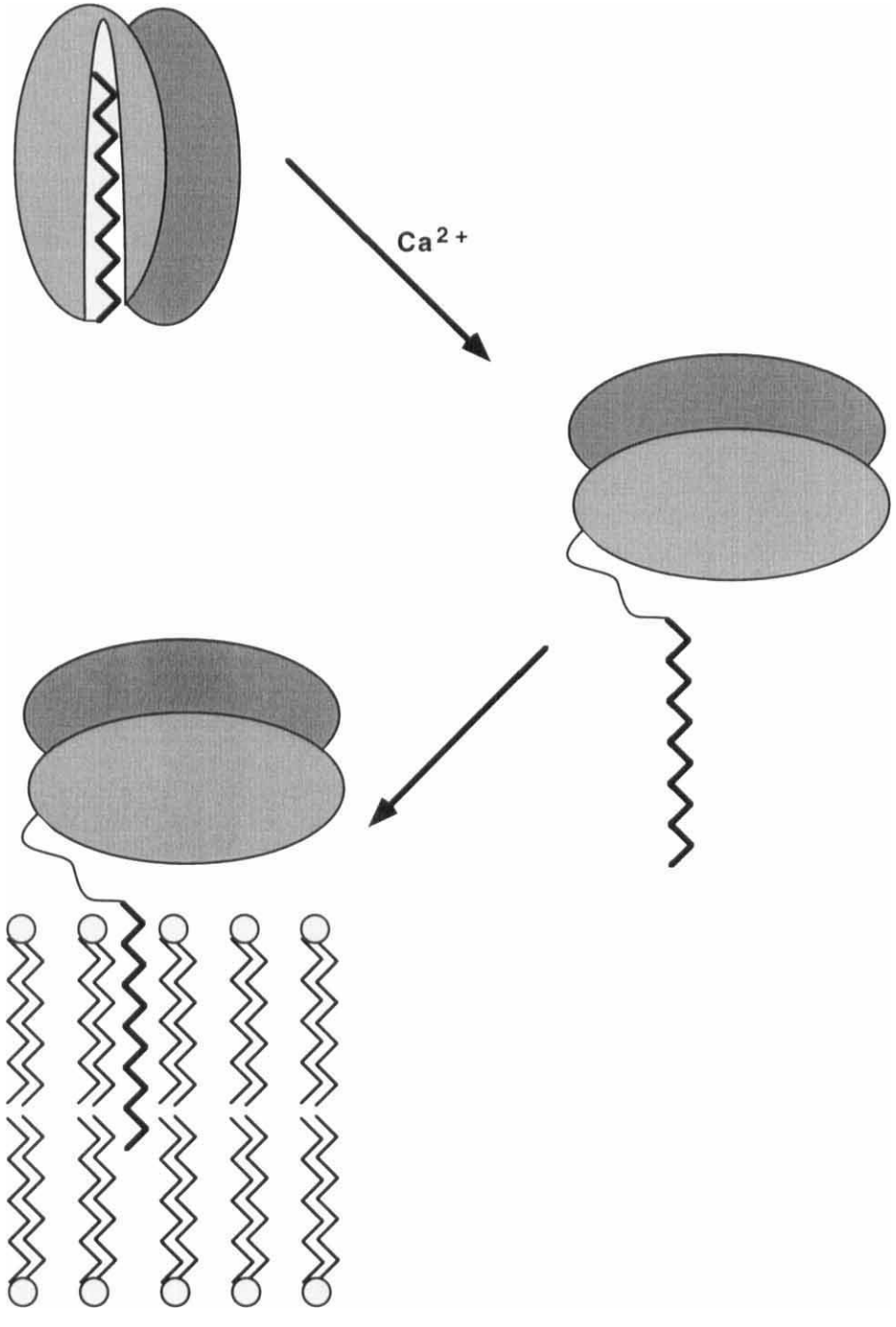
B. Recoverin

Recoverin is a protein of the EF-hand type described in Section II. However, as opposed to most EF-hand proteins, it is a membrane-associated protein that binds the intracellular face of membranes in a calcium-dependent manner. The protein is composed of four EF hands arranged pairwise, with only two of the four sites able to bind calcium (*83*). It is a member of the neuron-specific calcium sensor (NCS) family of proteins, which includes closely homologous proteins such as S-modulin and visinin, and less homologous proteins such as the vilip proteins or frequenin. The membrane binding is mediated by an acyl group, usually myristoyl, covalently attached to the amino terminus. In the apo form this myristoyl group is sequestered from solvent and released upon the binding of two equivalents of calcium (*84*). The structure of the unmyristoylated recoverin (*85*) was determined by X-ray crystallography and was followed by structures of the myristoylated apo form (*86*) and myristoylated calcium form (*87*). Together these structures paint the full pictures of the molecular transition between the inactive apo form and the active calcium form (Fig. 7). Briefly, in the apo state, the myristoyl group is buried in a deep hydrophobic pocket predominantly located in the amino-terminal EF-hand pair but with some contacts to the first EF hand in the carboxy-terminal domain. Upon calcium binding the hydrophobic pocket is closed and the myristoyl group is forced out. This is accompanied by a 45 degree rotation of the two EF-hand pair domains relative to one another. This type of calcium-induced conformational change is faintly reminiscent of that observed in calmodulin and troponin C but of opposite direction, (i.e., calcium binding closes a hydrophobic binding site in recoverin whereas it opens one in calmodulin).

Functionally, recoverin acts by binding to rhodopsin kinase and inhibits rhodopsin phosphorylation (*88*). It has also been observed to bind rod outer segment (ROS) membranes (*89*). The interaction with rhodopsin kinase does not require amino-terminal acylation, but binding to ROS membranes is acylation dependent, suggesting two distinct binding sites and/or binding modes on recoverin.

C. Annexins

The annexins form a group of mainly intracellular proteins with a Ca^{2+}-dependent binding to phospholipids. No clear physiological role of the annexins has so far been defined, but a wide range of biological functions have been suggested (*90–97*). Amino acid sequence analysis


Ca2+

indicates that the Ca^{2+} coordination in the annexin sites differs from that of the EF hand, even though both structures are dominated by an antiparallel four-helix bundle. The crystal structures have been determined for annexin V from humans (*98–101*), chickens (*102*), and rats (*103*, *104*) as well as for annexin I from humans (*105*). These structures all contain four domains with multiple Ca^{2+}-binding sites. The loop between helixes A and B contains a double site (AB and AB′), and the loop between helixes C and D contains a single site (CD). In the structure described by Seaton and co-workers (*103*, *104*) the four domains have very similar AB loops, RMSD = 0.39 Å, and the Ca^{2+} coordination is identical for all domains. The AB site carries a fully coordinated Ca^{2+} whereas the Ca^{2+} in the AB′ site has fewer ligands and only half the occupancy of the AB site. It therefore appears clear that the AB site is the stronger one of these two sites. The fact that the AB′ site is only partially occupied in the crystal is in agreement with Ca^{2+}-binding studies that have shown that the Ca^{2+}-binding is weak in the absence of phospholipid membranes compared to what it is in the presence of membranes, mM and μM, respectively (*106–111*). In the presence of Ca^{2+}, annexin V binds to phosphatidylserine (PS) and phosphatidylethanolamine (PE)–containing membranes, and shows a clear preference for PS (*112*). Annexin V, however, binds very poorly to phosphatidylcholine (PC) or phosphatidylinositol (PI), so charge is not the only determinant for this binding, because PE and PC are neutral whereas PS and PI are negatively charged at physiological pH. In a recent time-resolved fluorescence study (*113*) it was shown that the environment of the sole tryptophan in annexin V changes upon Ca^{2+} binding in the presence of phospholipid vesicles. The changes were interpreted to indicate that the tryptophan side chain moved from a hydrophobic to a more hydrophilic environment (i.e., from the hydrophobic core of the protein to the interface between the protein and the membrane). This interpretation is in good agreement with crystal structures of annexin V in the presence of glycerophosphoserine (GPS) or glycerophosphoethanolamine (GPE). These crystals were obtained from soaking annexin crystals with either GPS or GPE solutions and were found to bind only one GPS/GPE per annexin, namely to domain 3 (*104*). Due to the high degree of similarity of the four domains and the observa-

FIG. 7. A schematic diagram of the conformational change and extrusion of the myristoyl group from the N-terminal domain of recoverin upon calcium binding followed by insertion into the membrane.

tion by high-resolution electron microscopy that the annexin molecules are flattened on the membrane surface (*114*, *115*), it has been assumed that in solution all four domains will bind to the surface in a similar way (*104*). In both complexes a phosphoryl oxygen coordinates to the Ca^{2+} on the AB site (Fig. 8) replacing a water molecule. This will place the tryptophan side chain clearly inside the polar head group of the lipid and in level with the bond connecting the hydrocarbon chains with the glycerol moiety. These crystal structures will also explain the preference of PS over PE for annexin binding. The serine carboxylate coordinates the AB′ Ca^{2+} and the serine amino group forms hydrogen bonds to both the side chain of Thr187 and the main chain of Glu226. None of these interactions are present in the PE complex, and for the bulky PI and PC, which bind annexin poorly, repulsive steric interaction may be important. The crystal structure of annexin V/GPS clearly indicates that two Ca^{2+} in each domain are simultaneously coordinated to the protein and the lipid head group. This is the only direct evidence so far for this kind of interaction that has also been discussed for the membrane binding of C2 and Gla domains (discussed later). It is, however, also clear that there are many interactions that contribute to the binding (e.g., all four domains have a hydrophobic residue in the position analogous to the tryptophane in domain 3).

D. C2 Domains

The Ca^{2+}-binding C2 domain, first identified in various isoforms of mammalian Ca^{2+}-dependent protein kinase C (PKC) (*116–118*) is *ca.* 130 amino acids long. Because it was found that PKCs lacking the second domain did not show any Ca^{2+} regulation, it was proposed that the C2 domain was responsible for the Ca^{2+} regulation of PKC. Similar domains have been identified in various proteins such as synaptotagmin (*119–123*), cytosolic phospholipase A_2 ($cPLA_2$) (*124–126*), and phosphoinositide-specific phospholipase C, IP-PLC (*127–135*), among others. [For a recent review see (*136*).]

It has been shown that a single C2 domain from synaptotagmin will bind to phospholipid membranes in a Ca^{2+}-dependent manner (*119*, *120*). It has also been shown that this binding depends on the phospholipid composition (*121*). C2 domains from all the studied synaptotagmins (II–VI) bound to negatively charged phospholipids (phosphatidylserine, PS, and phosphatidylinositol, PI) in a Ca^{2+}-dependent manner. All but synaptotagmin IV were also found to bind to vesicles with a 1 : 1 mixture of negative and neutral phospholipids.

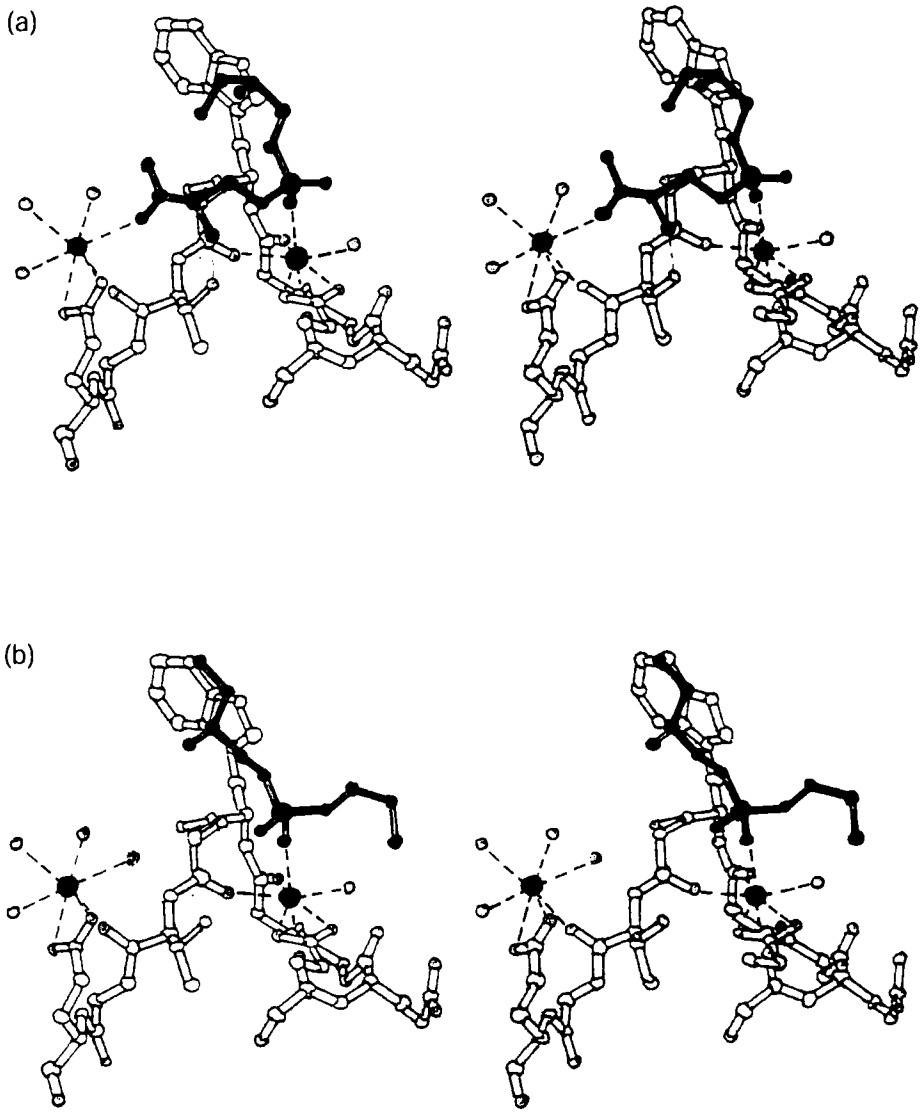


FIG. 8. Stereo view of the phospholipid binding site in the third domain of rat annexin V, including calcium ions in sites AB and AB′, (a) with bound glycerophosphoserine (GPS), and (b) with bound glycerophosphoethanolamine (GPE). GPS and GPE as well as the calcium ions are drawn dark. The oxygens in the protein fragment are drawn gray. (Redrawn from Swairjo *et al.* 1995).

None bound to only neutral phospholipid vesicles. The difference between IV and the other was localized to an Asp-to-Ser substitution and verified by mutant studies. Falke and co-workers (*137*) have shown that an isolated C2 domain from $cPLA_2$ binds Ca^{2+} and binds to phosphatidylcholine vesicles in a Ca^{2+}-dependent manner. The domain binds two Ca^{2+} in the absence as well as in the presence of phospholipid vesicles; however, the binding affinity increases from $K_D = 24\ \mu M$ in the absence of vesicles to $K_D = 3\ \mu M$ in the presence of vesicles.

In both instances the binding appears to be cooperative, resulting in a steep response to changes in Ca^{2+} concentration. The binding of Ca^{2+} to the C2 domain also increased the tryptophan fluorescence from Trp71, showing a conformation change caused by Ca^{2+} binding. The fluorescence increase was twice as large in the presence of vesicles as in the absence, showing that the tryptophan environment changes upon membrane binding. Falke and co-workers also studied the kinetics of the Ca^{2+} binding. It was thus found that in the phospholipid free state there is a single off rate for the two Ca^{2+}. This shows that either the two off rates are the same or the off rate for C2*Ca is much faster than that for $C2*Ca_2$. The latter could well be imagined for a system with strong cooperative Ca^{2+} binding and has been observed for EF-hand proteins (*62*, *138*). In the presence of vesicles, however, the Ca^{2+} off rate is much slower and there are clearly two off rates differing by a factor of 10 (Fig. 9). Very similar rates were obtained by measuring either the Ca^{2+} off-rate directly, the conformational change caused by the Ca^{2+} release, or the release of the C2 domain from the phospholipid surface. This therefore shows that the C2 domain with a single Ca^{2+} is still bound to the membrane surface, at least transiently. The equilibrium measurements, however, indicate that under conditions where, on average, one Ca^{2+} is bound per C2 domain, about half of the C2 domains will bind two Ca^{2+} and the other half will be in the apo form. It would be interesting to confirm this with a stopped-flow experiment designed in such a way that the final state will have a Ca^{2+}/C2 domain ratio of 1 : 1.

The Ca^{2+}-dependent binding of protein kinase C to phospholipid membranes has been shown to be specific for negatively charged phospholipids, and it has also been shown that Ca^{2+} binding depends on the presence of negatively charged phospholipids (*116*). The binding of PKC to phospholipid membrane depends not only on Ca^{2+} but also on the presence of diacylglycerol (*117*). This effect is very specific for PS-containing micelles. Even though PKC binds to phosphatidic acid–containing micelles, this binding is not affected by diacylglycerol. Fur-

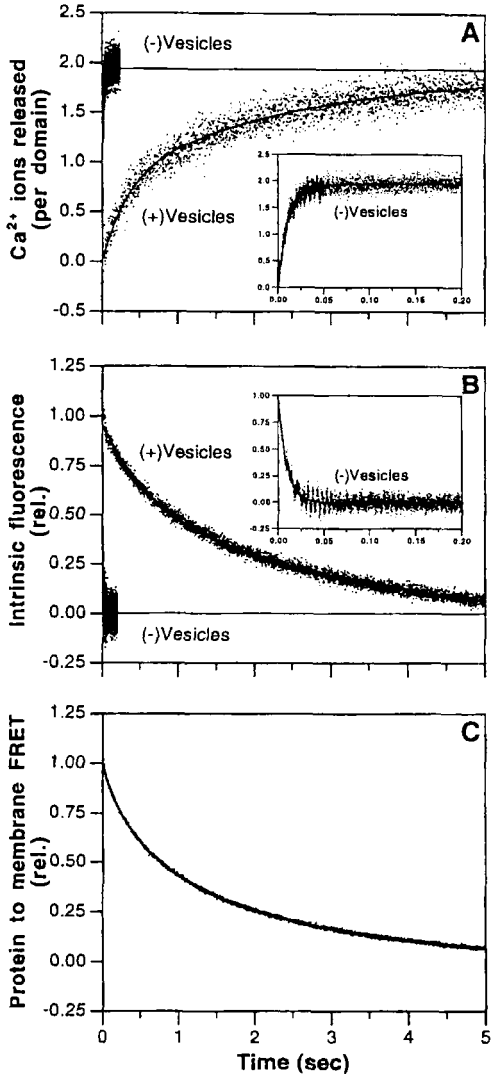


FIG. 9. Kinetics of Ca^{2+} dissociation from the $cPLA_2$ C2 domain in the absence and presence of phosphatidylcholine vesicles. (a) Stopped-flow measurement of the Ca^{2+} off rate using the fluorescent calcium indicator Quin-2. (b) Conformational changes triggered by Ca^{2+} removal from the C2 domain monitored using the intrinsic fluorescence of Trp71. (c) Membrane release of the C2 domain followed by protein-to-membrane FRET. (From Nalefski *et al.* 1997 with permission.)

thermore, only phosphatidyl-L-serine, and not phosphatidyl-D-serine, will cause the high-affinity binding of PKC to diacylglycerol-containing membranes (*118*).

The structure of the N-terminal C2 domain of synaptotagmin has been solved to a resolution of 1.9 Å for both the apo and Ca^{2+} forms (*139*). The structure is described as an eight-stranded β-sandwich with a conserved four-stranded motif named a C_2 key. The Ca^{2+}-binding site is found at the top of the β-sheet with ligands from four Asps in the loops connecting $\beta 2/\beta 3$ and $\beta 6/\beta 7$ (Fig. 10). A second possible but not occupied Ca^{2+}-binding site was identified in the other end of the molecule. Ca^{2+} binding to this domain caused only minor structural changes, indicating that the site is preformed. The structure of a C2 domain in an intact protein has also been determined for phosphoinositide-specific phospholipase C-δ_1 (*133–135*). The structure of PI-PLCδ1 comprises an N-terminal PH domain, a catalytic core, and a C-terminal C2 domain. Only the C2 domain will be further

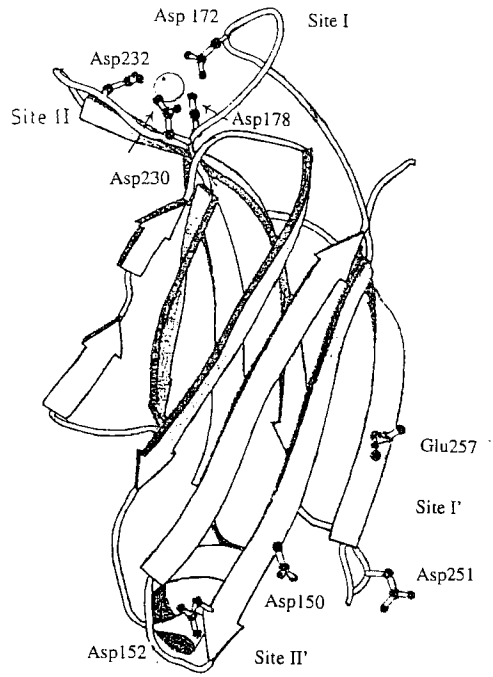


FIG. 10. Structure of the synaptotagmin C2 domain showing the calcium binding site at the top and ligands for a potential calcium binding site at the bottom. (From Sutton *et al.* 1995 with permission.)

discussed here. From a comparison of the structure of C2 domains from synaptotagmin and PI-PLCδ1, it is apparent that there are at least two different topological variants of the C2 domains (*135*). It is possible to go from the synaptotagmin topology to the PI-PLC topology by connecting the C terminus of β-strand 8 with the N terminus of β-strand 1 and cutting the loop between β-strands 1 and 2 open. This, however, appears to have no effect on the calcium-binding regions that are in the opposite end of the structure. Using lanthanum as a calcium analog showed that three loops form the metal-ion-binding sites with two adjacent metal ions. For another crystal form of PI-PLC, with samarium as a replacement for calcium, the same two sites were found (*134*). A comparison of the apo and lanthanide structures revealed only minor changes in the metal-ion-binding loops; however, the maximum distance between metal-ion-binding loops 1 and 2 changed from 12 Å in the apo form to 21 Å in the Sm^{3+} form (Fig. 11). Even though the functional role of the C2 domain in PI-PLC is still unknown, mutational studies have shown that it is essential for catalytic activity (*130*, *140*). The calcium-mediated binding of C2 domains to lipid membranes is still not well understood. The calcium-bridging model is to us very unattractive because it does not explain the specificity for PS. More studies are certainly needed, and our expectations are that a more specific membrane-binding model will eventually emerge, as it has for the annexins and the GLA domain in the vitamin K–dependent serum proteins (discussed later).

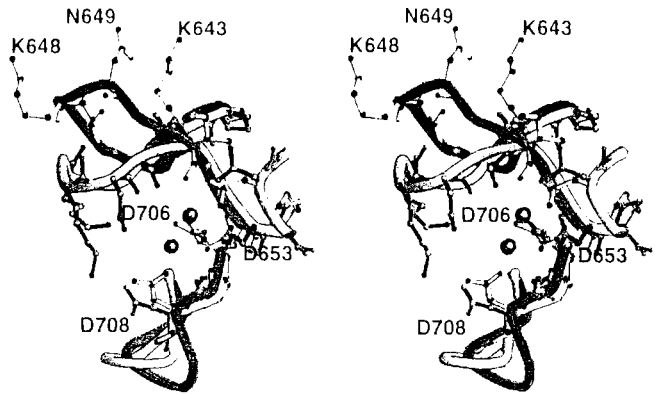


FIG. 11. Stereo view of C2 jaws of the apo and Sm^{3+} forms of PLC-δ1. The apo form is shown with dark backbone and light side chains, and the Sm^{3+} form with light backbone and dark side chains. (Redrawn with permission from Grobler *et al.* 1996.)

E. γ-Carboxyglutamic Acid Sites

The amino acid γ-carboxyglutamic acid (Gla) is not one coded by DNA, but glutamic acid is posttranslationally carboxylated. Gla contains a malonic acid moiety with an affinity for Ca^{2+} of $K_b = 30\ M^{-1}$. This is too weak to be of any biological relevance by itself. However, many of the modified amino acids often appear together, though not necessarily in sequence, as in the case of the Gla module of blood coagulation factors, in which there are *ca.* 10. Robertson *et al.* (*141*) have shown, by using ^{43}Ca NMR, that calcium binding to a Gla-Gla moiety is sufficiently strong to be relevant under physiological conditions ($K_D = 0.6$ mM). Likewise Colpitts and Castellino (*142*) have found a $K_D = 1.6$ mM for calcium binding to the peptide CysIleGla-GlaIleCys but with a stoichiometry of two peptides per calcium ion. Later, we will discuss the Gla domain from the blood coagulation system at some length and, to a minor degree in Chapter 4, the Gla-containing conotoxins, whereas for the bone Gla and matrix Gla proteins (*143*) the reader is referred to older reviews (*144*).

The Gla-containing proteins of the blood coagulation system are all modular with the Gla-domain N terminal. Factors VII, IX, and X and protein C form a group with the same domain structure. The Gla domain is followed by two EGF-like domains, of which the first one also binds calcium (see Section IV.C), and a serine protease domain, also with a calcium-binding site (see Section IV.D). Prothrombin and protein S have somewhat different domain structures (*145*, *146*). In prothrombin the Gla domain is followed by a hexapeptide with a disulfide loop, two kringle domains, and the C-terminal serine protease domain. In protein S the Gla domain is followed by the "thrombin-sensitive" loop, four EGF-like domains, and the C-terminal domain that is homologous to plasma steroid hormone–binding proteins.

The DNA sequence for the Gla domain show that there should be 9–12 Glu residues. These Glu residues are all carboxylated to Gla by a vitamin K–dependent carboxylase. This modification is a prerequisite for calcium binding and biological activity. The first attempts to characterize the calcium binding properties of Gla domains were made in the early 1970s (*147–149*). These studies were made before the Gla residue had been identified (*150*, *151*). Since these early works, the calcium-binding properties of various Gla domains have been reported on several occasions. In most cases equilibrium dialysis experiments with ^{45}Ca have been used, but spectroscopic techniques as well as calorimetry and ion-selective electrodes have also been em-

ployed (*152–163*). Equilibrium studies have indicated that all vitamin K–dependent plasma proteins bind 6 to 12 calcium ions (*159*). The low affinity of these calcium binding sites (K_D between 0.1 and 2 mM) and their large number have made the determinations difficult. In two recent studies (*164*, *165*) on synthetic peptides with sequences corresponding to the N-terminal domain(s) of factor IX and protein C it has been shown that it is not sufficient with only the Gla domain [factor IX(1–42) and protein C(1–38)] for full calcium binding. However, including the amino acids from the hydrophobic stack [factor IX(1–47), protein C(1–48)] seems to restore both calcium and lipid binding up to the level in intact proteins. Even though the sequences of these peptides are 50% identical, there seem to be some important differences regarding calcium and lipid binding of the truncated peptides. Removing the five C-terminal residues from factor IX(1–47) drastically decreases calcium binding as well as the binding to phospholipid vesicles, whereas for protein C(1–38) the calcium binding has been reduced merely by a factor of two to three, and full binding to phospholipid vesicles can be obtained by a modest increase in the calcium concentration. It is at present not clear what causes this difference, but it seems to reside in the first 30 amino acids, because a peptide with the first 30 amino acids from the human protein C sequence and the remaining eight residues from the factor VII sequence showed a calcium-binding pattern similar to the 38-mer with the protein C sequence (*142*). Culpitts *et al.* (*166*), using synthetic protein C peptides selectively ^{13}C labeled on individual Gla residues, have been able to show that Gla 6, 16, 25, and 26 (factor X numbering) participate in the stronger calcium binding.

Even though it seems clear that it is sufficient with the Gla domain and the hydrophobic stack to restore full calcium-binding activity, the apo form of these peptides appears essentially structureless (*167*), whereas the fragment from factor X (also comprising the N-terminal EGF domain) has a more ordered structure (*168*). It has also been shown recently that the dynamic properties of Trp42 in the apo form of factor X peptides critically depend on the length of the peptide (Drakenberg, unpublished results). The correlation time of Trp42 is lengthened *ca.* threefold (from 1.5 to 4.5 ns) by extending the 46–amino acid peptide with either the EGF domain or five amino acids from the C-terminal helix from calbindin D_{9k}. It is thus clear that even though the Gla domains of factor IX and protein C do not need the EGF domain for full calcium-binding properties, the apo-Gla domain of factor X needs the EGF domain to stabilize the structure. The structure of the apo form of the Gla domain from factor X, in the

GlaEGF fragment, has all the secondary structure found in the calcium form (discussed later); however, the relative orientations of the structural elements differ, especially in the N terminus. In this structure the Gla residues are all, as could be expected, exposed to the solvent, and the hydrophobic residues Phe4, Leu5, and Val8 form a cluster facing the interior of the Gla domain [Fig. 12(a)].

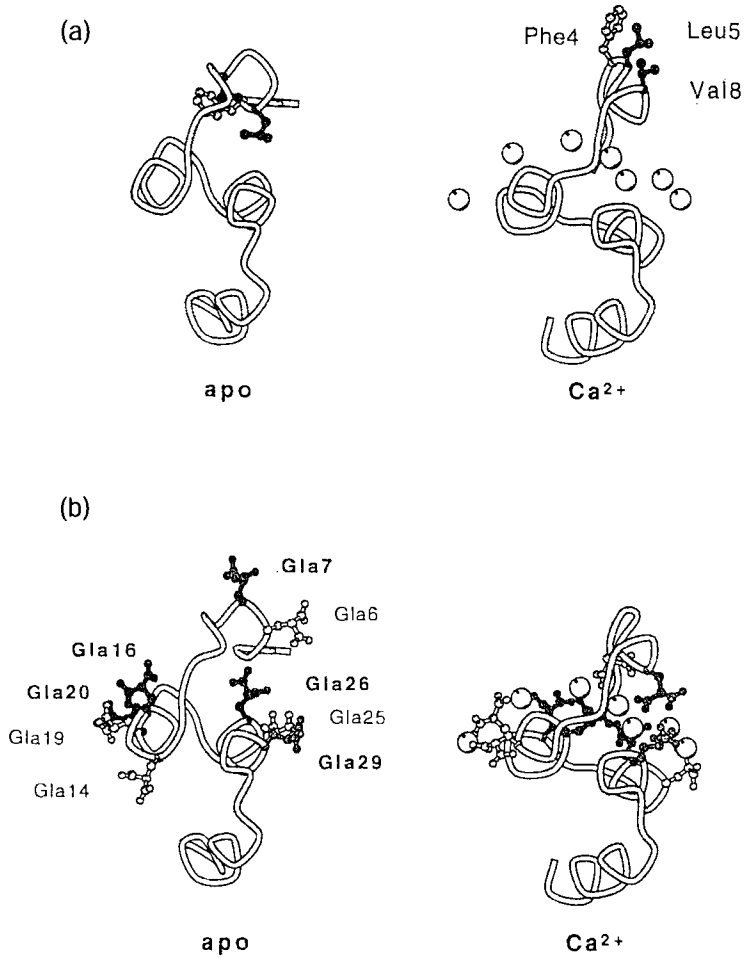


FIG. 12. Comparison of the energy-minimized average NMR structure of the Gla domain from factor X with model-built calcium-loaded Gla domain (based on the X-ray structure of prothrombin). (a) Location of residues Phe4, Leu5 and Val 8. (b) Location of Gla residues 6, 7, 16, 19, 20, 26, and 29. The essential residues 7, 16, 20, 26, and 29 are shaded dark.

The structure of calcium-saturated Gla domains has been determined both in the crystal state by X-ray crystallography for prothrombin fragment 1 (*169*) and for factor VII in complex with tissue factor (*170*) and in solution by NMR for the N-terminal 47 amino acids from factor IX (*171*, *172*). These three structures are essentially the same and are shown in Fig. 12(b). The main secondary structure elements of the structures are three helixes (14–17, 25–31, and 36–47) and an N-terminal loop. Seven Ca^{2+} are identified in the structure, and they have coordination numbers ranging from three to seven. Five of these Ca^{2+} are chelated between Gla6, Gla7, Gla16, Gla25, Gla26, and Gla29, rendering four of them inaccessible to water. In the NMR structure determination there is no direct information on the position of the Ca^{2+}; however, in a refinement they used a genetic algorithm to identify the locations of the Ca^{2+} (*172*). The identified Ca^{2+} positions agreed well with the crystallographically determined positions and were subsequently used in the refinement of the solution structure. The two crystal structures of the Gla domains are very similar, even though they are in completely different settings, with an RMSD of 0.63 Å for 40 α-carbons. A similar comparison of the solution structure of factor IX and the prothrombin crystal structure reveals an RMSD of 1.3 Å for residues 12–47. The backbone conformation for the 11 N-terminal residues differs significantly between the solution and crystal structures. All Gla domains except that in human factor IX have an Ala as the N terminus. In factor IX it is a Tyr. This larger residue cannot be accommodated within the crystal structure, which may explain the difference between the crystal and solution structures in this region. However, the unusual solvent mixture with 3 M urea and 2.5 M guanidine hydrochloride used in the NMR study may also cause some structural changes. Based on these structures and the structure of the apo-Gla domain from factor X determined by NMR (*173*), we can now speculate about how the Gla domains are bound to phospholipid membranes (*122*, *174*, *175*). It can be assumed that the conserved hydrophobic residues, Phe4, Leu5, and Val8 (human factor X numbering) are important for the membrane binding because they are exposed on calcium binding (Fig. 12). Also, based on site-specific mutagenesis studies of Leu5, it has been suggested that this side chain is bound into the phospholipid membrane. The hydrophobic binding is certainly not sufficient to explain all experimental data on the membrane binding. For optimal binding the membrane must contain negatively charged head groups, phosphatidylserine. This clearly indicates that also electrostatic effects are important for the membrane binding, and Li *et al.* (*122*) argue that some

of the peripheral calcium ions in the Gla domain as well as the side chains of Lys3, Arg9, Lys10, and Arg15 in human prothrombin may be directly involved in the interaction with the membrane. However, only for residues 9 and 15 is the charge conserved in the Gla domains, so we may argue that they are strong candidates for direct interaction with the negatively charged lipid head groups.

IV. Extracellular Calcium-Binding Proteins

A. Introduction

The total concentration of calcium in the blood plasma is *ca.* 2 mM, and about half of it is bound to proteins, mainly serum albumin. This high calcium concentration is typical for all extracellular fluids, in stark contrast to the very low free calcium concentration in resting cells. One might therefore be led to believe that there is no specific function of calcium in the extracellular fluids. This is certainly not true. It has been known for almost a century that calcium is critical for blood coagulation, and it is also well known that calcium is a major component in our skeleton. It is, however, also obvious that the requirement for a protein to be calcium binding in a milieu with free calcium at 1 mM is very different from one with calcium at μM levels.

B. Conantokins

Cone snails produce biologically active peptides to paralyze their prey. The peptides are known as conotoxins (*176–178*). Most of them are small and stabilized by disulfide bonds. Two unusual members of the conotoxin family are conantokin G from *Conus geographica* (*179*) and conantokin T from *Conus tulipa* (*180*), which lack disulfide bonds but are rich in γ-carboxyglutamic acid residues:

con-G: GlyGluGlaGlaLeuGlnGlaAsnGlnGlaLeu Ile ArgGlaLysSerAsn
con-T: GlyGluGlaGlaTyrGlnLysMetLeuGlaAsnLeuArgGlaAlaGluValLysLysAsnAla

The role of the Gla residues is not yet fully understood, but a synthetic conantokin peptide with Glu instead of Gla has been shown to be inactive. However, it is still not clear whether Ca^{2+} is essential for biological functioning of the conantokin peptides (*181*, *182*). There appears to be no thorough study of the calcium ion binding to these peptides, though CD spectroscopy has been used to study the change in helical content as a function of calcium concentration (*183*). The

two peptides behave quite differently. con-G had almost no helical structure in the calcium-free state but it increased to *ca.* 50% in the presence of 9 mM Ca^{2+}. con-T on the other hand already had roughly 50% helix without Ca^{2+} added, and this did not change upon addition of calcium. The same effect was found with Cu^{2+} whereas Mg^{2+} and Zn^{2+} were found to introduce almost 70% helix in con-G (Fig. 13). Another interesting observation is that *ca.* half of the calcium-induced helix in con-G remains in the presence of excess EDTA. The authors have interpreted this as being due to a very strong calcium ion binding to con-G; however, we also have to consider that EDTA has been shown to interact with various proteins in a calcium-dependent manner (*184*). We therefore find a more likely explanation that the Ca^{2+}-EDTA binds to con-G and introduces some helical structure. Skjaerbaek *et al.* (*183*) have also determined the solution structure of con-G in the presence of calcium, and it appears to be more helical than in the absence of calcium. However, the resolution is not sufficient to define binding sites for calcium, making the presence of a site strong enough to compete with EDTA for calcium less likely. Rigby *et al.* have determined the solution structure of con-G to high resolution in the absence (*185*) as well as in the presence of Ca^{2+} (*186*). The peptide is well structured under both conditions, with backbone RMSDs of 0.8 and 0.6 Å, respectively. As shown in Fig. 14, there is a pronounced structural change upon Ca^{2+} binding, with the formation of a straight α-helix from Gla3 to Lys15. In this structure Gla residues 3, 7, 10, and 14 become aligned in a linear array on one side of the helix. The Ca^{2+}, which cannot be "seen" by NMR, were localized with a genetic algorithm (*172*). The best model was obtained with 4 Ca^{2+}/peptide. All the Ca^{2+} were found to have 3 or 4 ligands from the Gla side chains. This arrangement has some similarity to the Ca^{2+} binding to the Gla domains in blood coagulation proteins (*170*, *187*), in which there also is a linear array of Ca^{2+} coordinated to Gla side chains. In both cases the calcium binding will also expose some hydrophobic amino acids to become solvent accessible.

C. EGF-Like Domains

The epidermal growth factor (EGF)–like domains are approximately 45 amino acids long and contain six cysteine residues that are paired in a characteristic manner, 1 to 3, 2 to 4, and 5 to 6, with a double-stranded β-sheet as the main structural feature. The EGF domain has been found in a wide variety of proteins including these involved in blood coagulation, fibrinolysis, neuronal development, and

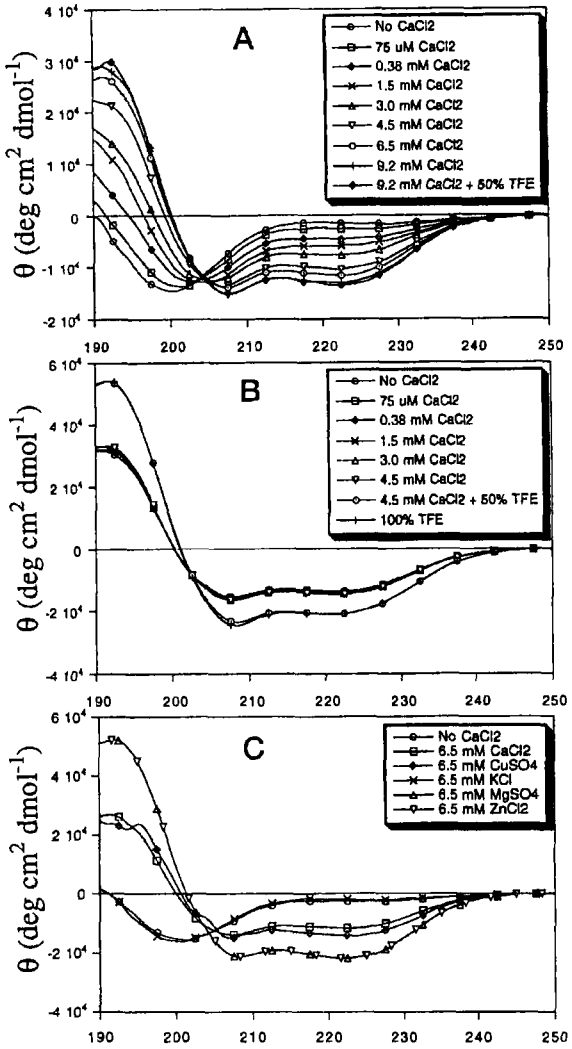


FIG. 13. CD spectra of con-G and con-T under different conditions. (a) CD spectra of con-G at different $CaCl_2$ concentrations. (b) CD spectra of con-T at different $CaCl_2$ concentrations. (c) CD spectra of con-G with different metal ions. (d) CD spectra of con-T with different metal ions. (e) CD spectra of con-G with fixed $CaCl_2$ concentration and different EDTA concentrations. (From Skjaerbaek *et al.* 1997 with permission.)

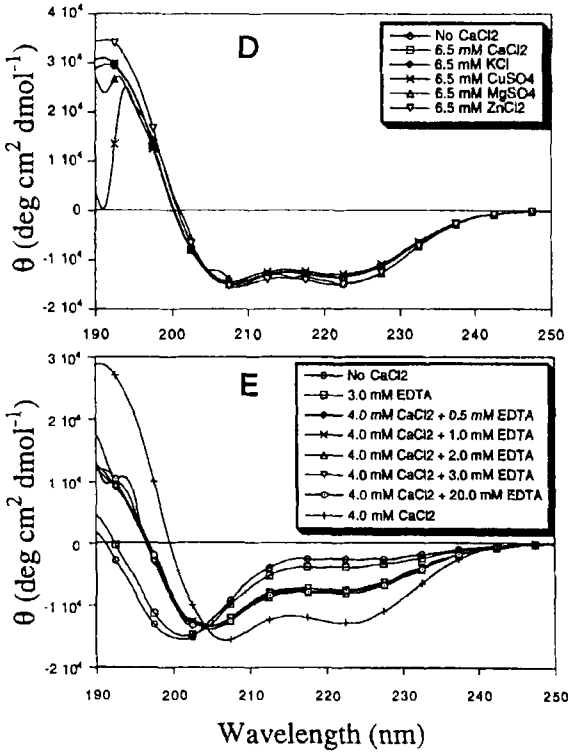


FIG. 13. (*continued*)

cell adhesion (*188*). A subset of these domains has a hydroxylated aspartic acid (Hya) or hydroxylated asparagine (Hyn), most prominently found in vitamin K–dependent plasma proteins with an identified consensus sequence of Cys-X-Hya/Hyn-X-X-X-X-Tyr/Phe-X-Cys-X-Cys (*189*, *190*). The consensus sequence for Asp/Asn hydroxylation has also been found in EGF domains from, for example, fibrillin, which has 46 EGF domains whereof 43 have the consensus sequence for hydroxylation (*191–193*); fibulin-1, in which four of nine EGF domains have the consensus sequence (*194–197*); and the Notch receptor, in which 21 of 36 EGF domains have the consensus sequence for hydroxylation (*198*). [For a review see (*188*).] The presence of the hydroxylated amino acid has also been correlated with the presence of a Ca^{2+}-binding site with a K_D in the range of 10 to 100 μM (*199–204*). Studies of Ca^{2+}-binding properties of a protein fragment from protein C containing the two EGF domains demonstrated that this fragment contains one Ca^{2+}-binding site (*205–207*). This site was fi-

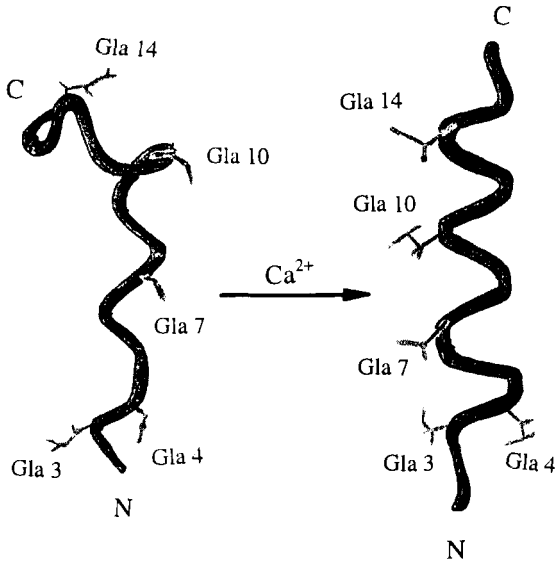


FIG. 14. Calcium-induced transition in con-G with the apo form to the left and the calcium form to the right. (From Rigby *et al.* 1997 with permission.)

nally localized to the N-terminal EGF domain in protein C as well as in factors IX and X (*206*, *208*, *209*).

The isolated EGF domains have been shown to bind Ca^{2+} with low affinity (K_D, 0.5–5 mM) (*206*, *208–212*); however, these domains usually appear at least in pairs and often several in tandem or as in the vitamin K–dependent serum proteins with a Gla domain bound N-terminally. It has been shown that when the N-terminal domain is intact, the Ca^{2+} affinity to the next domain has increased by approximately 10-fold, to K_D = 0.1 mM, making these sites essentially saturated under physiological conditions (*162*, *213*, *214*). Similarly, Handford and co-workers (*211*, *215*) have found that a recombinant fragment of fibrillin comprising a pair of EGF domains has a high affinity (K_D = 0.35 mM) and a low affinity (K_D = 9.2 mM) site. The increased affinity to the C-terminal domain is explained as caused by the donation of a ligand from the N-terminal EGF domain to the site of the C-terminal EGF domain. The Ca^{2+} binding to the high-affinity site is in good agreement with a value of 0.25 mM reported as the average affinity to a proteolytic fragment of fibrillin containing seven intact EGF domains (*193*). Protein S has four EGF domains in tandem, and it has been shown that the Ca^{2+} affinity to this protein is much stronger (K_D, 10^{-9} to 10^{-7} M) than for other EGF-containing

proteins studied so far (*216*). There is no obvious sequence variation to explain this extremely strong Ca^{2+} binding, and the isolated EGF domains from protein S do not display any extreme Ca^{2+} affinity (*217*). However, fragments containing various numbers of EGF domains from protein S showed a very interesting Ca^{2+} affinity (*218*). Extremely strong Ca^{2+} binding was thus found for fragments containing EGF1–4 and EGF2–4 ($K_D < 10^{-6}$ in physiological salt) whereas hundredfold higher K_d's where found for fragments not containing EGF4. Another noteworthy observation is that EGF3 has a 350-fold higher affinity for calcium in fragment EGF2–4 than in fragment EGF1–3. It has been shown on several occasions that the Ca^{2+} affinity to an EGF domain is reduced significantly if the protein segment N-terminal to it is removed. This may be an effect of stabilization of the site, either by having a calcium ligand in this part of the protein or just by stabilizing the conformation of the calcium site. The data reported on the site in EGF3 of protein S demonstrates an influence on Ca^{2+} binding from a domain on the C-terminal side. This indicates that the three-dimensional structures of these fragments are such that there are direct interactions between nonadjacent domains and not a linear array of domains. Another protein displaying an unusually high calcium binding to EGF domains is Notch-1, a fundamental receptor for cell fate decisions (*219*). This protein has 36 EGF domains, of which 21 have the consensus sequence for calcium binding. Calcium binding studies to the recombinant fragments comprising EGF11–12 and EGF10–13 have shown that this protein has an calcium affinity in the low μM range and thus somewhere in between protein S and the other calcium-binding EGF domains.

Although the role of Asp/Asn hydroxylation on calcium binding has been a matter of debate, there have been very few attempts to resolve this question. Morita and Kisiel (*200*) compared the calcium affinity of bovine and human factor IX. The calcium affinities were shown to be similar even though the degree of hydroxylation is different for these two proteins. The conclusion was uncertain, however, due to interference with other calcium-binding sites in the proteins. Later Selander Sunnerhagen *et al.* (*210*) showed, using single EGF domains, that even though there is a small effect due to hydroxylation, it is not significant, so what might be the role of the hydroxylation is still an open question.

The structures of several isolated EGF domains have been determined by NMR, the first one more than 10 years ago (*220–232*). These structures are all quite similar, revealing a domain structure consisting of two relatively independent subdomains. The N terminal

constitutes about two thirds of the domain, and its major structural motif is a β-sheet with two or three strands. The C-terminal third of the domain has been described as a small antiparallel double-hairpin structure (*231*) or as two loops connected by a short β-sheet and a flexible C terminus (*224*). The three-dimensional structures of EGF domains have also, more recently, been determined by X-ray crystallography (*170*, *233–236*). Only a few of these studies deal with EGF domains of the Ca^{2+}-binding type (*170*, *235*, *236*). In the following we will restrict the discussion to the EGF domains of the Ca^{2+}-binding type (cbEGF).

The solution structure of the N-terminal cbEGF domains from factor IX (fIX-EGF_N) and factor X (fX-EGF_N), both containing Hya, in the absence of calcium, has been determined by means of 2D NMR (*227–229*). The structure of fX-EGF_N has also been determined in the presence of calcium (*230*). Even though the calcium ion cannot be "seen" by NMR, the calcium site can be readily identified (Fig. 15). In the calcium form of fX-EGF_N there is a cavity of appropriate size for a calcium ion that is lined with oxygens oriented toward the center. There are five well-defined ligands, two side-chain carboxylate groups (Asp46 and Hya63), one side-chain carbonyl group (Gln49), and two

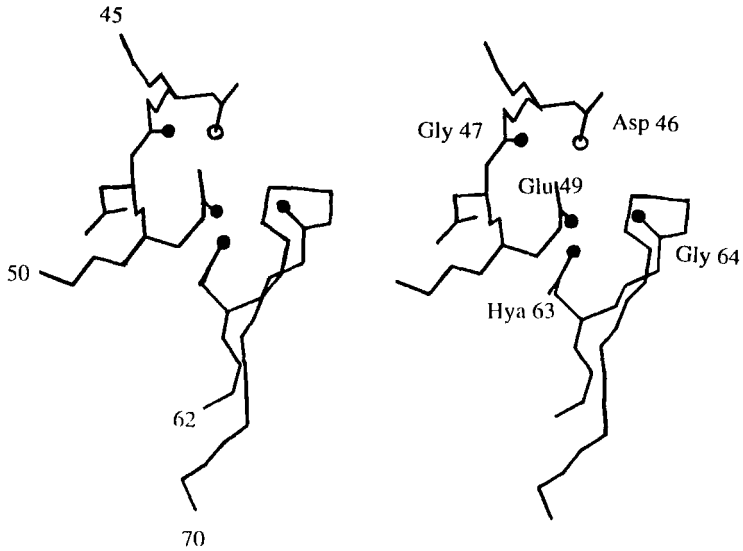


FIG. 15. Stereo view of the calcium-binding site of fX-EGF_N. Amino acids unambiguously assigned as calcium ligands are labeled and the coordinating oxygens are marked with filled symbols (Gly47, Gln49, Hya63, and Gly64). Asp46, a potential ligand, is marked with an open circle.

backbone carbonyls (Gly47 and Gly64). This was later confirmed in two crystallographic studies on the N-terminal EGF domain of factor IX (*235*) and for the N-terminal EGF domain in factor VII in complex with tissue factor (*170*). Furthermore, the side chain of the Hya residue is rotated in such a way that the hydroxyl group cannot be a ligand to the calcium ion (*230*). The changes in the NMR spectra caused by calcium binding are localized to residues 46–51 and 62–68, and are not very dramatic. A comparison of the structures of the apo and calcium forms of fX-EGF_N shows no major differences. There are, however, some minor differences. The N terminus is more well defined in the calcium form and has moved slightly toward the main β-sheet. The turn connecting the two strands in this β-sheet is bent somewhat toward the N terminus. These conclusions are confirmed by the X-ray crystal structure of fIX-EGF_N with calcium (*235*), and the backbone RMS deviation between the two structures is 1.2 Å (*237*).

There are now also available structures on the Gla–EGF pair from factor X (*168*) and on an EGF pair from fibrillin (*237*), both in solution and solved by NMR in the presence of calcium. Information on the function of calcium in this kind of structure is still very limited. Only for the Gla–EGF pair is information available for both the apo and calcium-loaded states. Even though the structures of the individual domains are reasonably well defined, the relative orientation, which is probably more interesting, is not. It is, however, from NMR as well as SAXS data (*168*), clear that the relative orientation of the two domains is calcium dependent. In the apo state there is a more or less linear arrangement of the two domains, whereas in the calcium form there is a more compact structure, with the two domains oriented almost perpendicular to each other (Fig. 16). This orientation, however, does not agree with what was found in the crystal state for the complex between factor VII and tissue factor (*170*), where the Gla and the N-terminal EGF domain are arranged in a more or less linear manner. We have to consider, though, that these are different proteins, though quite similar, each with its own specific interactions. Based on the crystal structure of the isolated cbEGF domain of factor IX, it was speculated that an N-terminal domain might donate a ligand to the calcium site in the following domain (*235*). In none of the structures determined so far with two domains and a calcium-binding site in the C-terminal domain has such a ligand donation been observed. More recently Downing *et al.* (*237*) have found from sequence analysis of domain pairs indications that they can be divided into two classes based solely on the number of residues linking the two do-

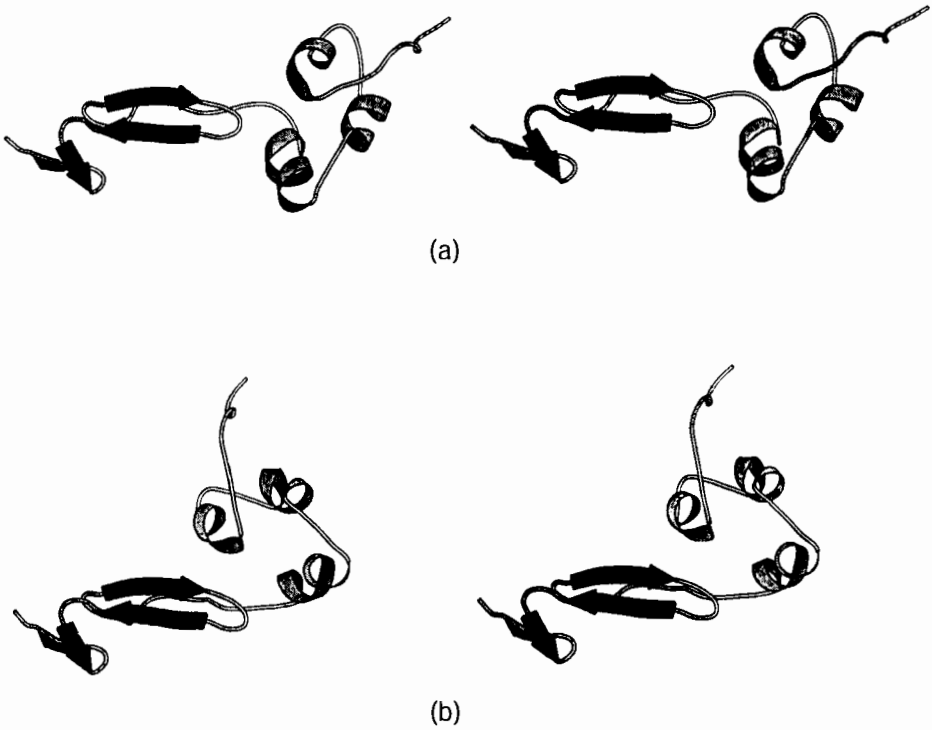


FIG. 16. Stereo representation of the secondary structure elements and relative orientation of the domains in the Gla–EGF domain pair. (a) with 1 equivalent of Ca^{2+} added and (b) in the absence of Ca^{2+}. (From Sunnerhagen *et al.* 1996 with permission.)

mains. Sequence alignment of the two classes of pairs was applied to define the consensus sequences as shown in Fig. 17. They also suggest that the Class II pairs are likely to adopt a different conformation and that a conserved carboxylate/carboxamide side chain may donate a ligand to the following domain, as is observed in the crystal structure of fIX-EGF_N. The confirmation of this hypothesis has to await the structure determination of such a domain pair. This hypothe-

Class I

EGF1: Dx D/N ECxxxxxxCxxxxxCxNxxGS Y/F xCxCxxG Y/F xxxxxxxxC
EGF2: xD I/V D/N ECxxxxxxCxxxxxCxNxxGS Y/F xCxCxxG Y/F xxxxxxxxC

Class II

EGF1: xx D/N xCxxxPCxNG G/A xCxxxxxx Y/F xCxCxxG Y/F xGxxC
EGF2: xx D/N I/V D/N E/D CxxxPCxNG G/A xCx D/N x I/V xx Y/F xCxCxxG Y/F xGxxC

FIG. 17. Consensus sequences for class I and class II EGF–cbEGF and cbEGF–cbEGF pairs. x indicates that there is no preferred amino acid and letters separated by / indicates that both amino acids are commonly present.

sis may explain the high calcium affinity in the fragments from Notch studied by Rand *et al.* (*219*) with K_D in the low μM range even in the presence of physiological salt concentration. For protein S on the other hand, this does not offer any explanation for the high affinity because the protein S pairs with high affinity belong to Class I (*218*).

D. Serine Proteases

It has long been known that some serine proteases contain one or two calcium-binding sites. It has thus been shown that trypsinogen has two sites, one of low affinity in the activation peptide and one with a higher affinity ($K_D = 1.6 \cdot 10^{-5}$ M) that is also present in the active enzyme (*238*, *239*). The structure of trypsin with one bound calcium has been determined to a resolution of 1.8 Å, and the calcium ligands could be identified (Fig. 18) (*239*). The calcium ion is coordinated by six ligands at the edges of an octahedron. Four ligands are from a loop of the protein, Glu70–Glu80, where Glu70 and Glu80 are coordinating with their side-chain carboxylate groups and Asn72 and Val75 are coordinating with the backbone carbonyls. The two remaining ligands are water molecules that are also hydrogen bonded to Glu70 and Glu77, respectively. Sites similar to this archetype can be inferred from the sequences of chymotrypsin; blood coagulation factors VII, IX, and X; and protein C (*184*, *240–243*). For trypsin, chymotrypsin, and their zymogenes it has been shown, using ^{43}Ca NMR and stopped-flow experiments, that the Ca^{2+} exchange is fairly slow even though the binding affinity is modest (Fig. 19) (*184*). The on rate of the calcium ion was thus found to be 10^5–10^6 s^{-1}, two to three orders

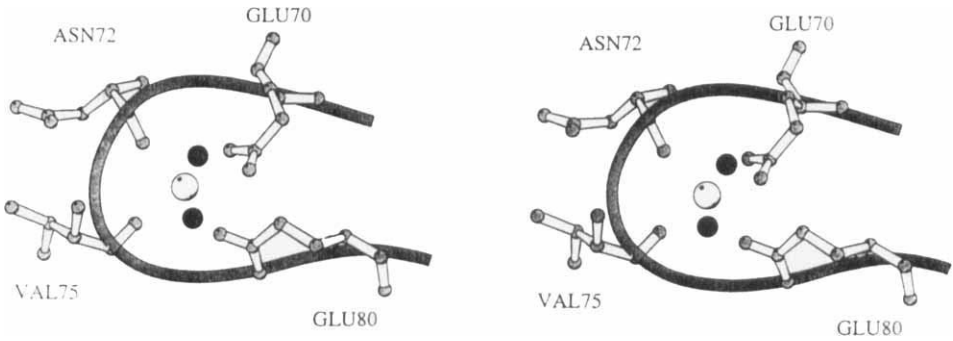


FIG. 18. Stereo view of the calcium-binding loop in trypsin including the calcium ion and internal water molecules.

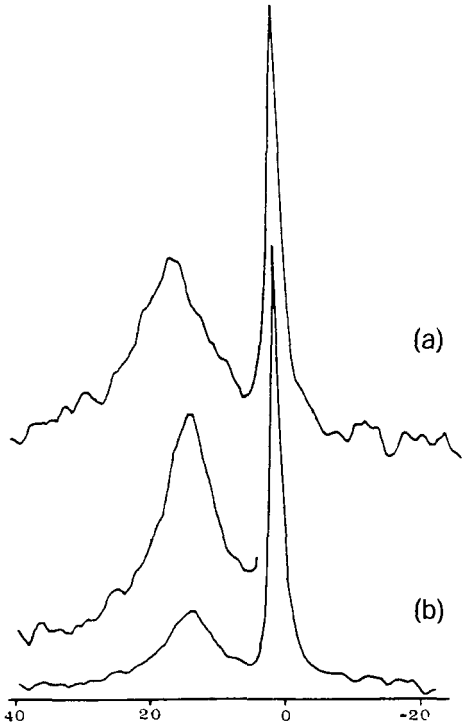


FIG. 19. ^{43}Ca NMR spectra of (a) 2 mM tosyl-trypsin and 2.5 mM Ca^{2+} at pH 6.5 and 24°C; (b) 1 mM tosyl-trypsinigen and 1.3 mM Ca^{2+} at pH 6.2 and 24°C.

of magnitude slower than what could be expected for a diffusion-limited process. Also, the off rate from chymotrypsin(ogen) is at least one order of magnitude faster than that from trypsin(ogen). These findings are in agreement with the reported higher flexibility in the calcium-binding site in chymotrypsin apparent in the crystal structure. However, the crystal structures do not offer any explanation of the slow calcium-ion exchange because the binding site is close to the surface and there are two water ligands.

The calcium-binding sites in the protease domains of coagulation factors VII, IX, and X and protein C all have calcium affinities comparable to that in trypsin (*241*, *244–246*). In the crystal structures of factors IX and X, no calcium ions could be identified, most likely due to the crystallization conditions because the calcium-binding site is present though not occupied (*234*). The crystal structure of factor VII in complex with tissue factor shows the presence of calcium in the

serine protease domain with a structure very similar to the one in trypsin. Calcium binding to the serine protease domain of protein C has a pronounced effect on the activation of protein C by thrombin. This activation is very slow in solution and is reduced even further in the presence of calcium. However, if protein C is activated by the thrombin–thrombomodulin complex, the activation is rapid and is increased in the presence of calcium (*234*). Calcium binding to the serine protease domain of coagulation factor VII is important both for amidolytic activity and for interaction with the tissue factor (*247*).

The subtilisins all have a calcium-binding site whose occupation protects the enzyme against autolysis (*248*). This site is very different from the one in trypsin, however. The ligands come from three different parts of the protein, and the calcium binding seems to bring the N terminus close to the 75–81 and 40–42 loops with coordination to the side chains of Gln2 and Asp41, and to the peptide carbonyl of Leu75, Asn77, Gly79, and Val81 (*249–251*).

E. α-Lactalbumin and Lysozymes

α-Lactalbumins (aLACs) are milk proteins that play an important role in the biosynthesis of the milk sugar lactose. By binding to the enzyme galactosyltransferase (GT), the lactose synthase complex is formed. This binding is reversible, and aLAC functions as a regulatory subunit. GT alone is unable to catalyze the synthesis of lactose at physiological glucose concentrations due to its low affinity for glucose. In the lactose synthase complex the affinity for glucose has increased 1000-fold and lactose can be produced (*252–254*). On the other hand are the lysozymes (LZs), lytic enzymes that catalyze the degradation of peptidoglycans. From the high degree of homology between aLAC and LZ as revealed by amino acid sequences (*255*, *256*), intron–exon arrangements (*257*, *258*), and three-dimensional structure (*259*, *260*), it has been postulated that these functionally different proteins have evolved from a common ancestral molecule. It is recognized that all known aLACs possess one high-affinity Ca^{2+}-binding site, and crystal structures have revealed that the Ca^{2+}-binding site in aLAC is formed by the side-chain carboxylate groups from three aspartate residues (Asp82, Asp87, and Asp88), which are conserved in all aLACs. Also, the backbone carbonyls from Lys79 and Asp84 contribute to the ligation of Ca^{2+} as well as two water molecules (*259*, *260*). Ca^{2+} binding to lysozymes from horse and pigeon with the conserved aspartate residues required for Ca^{2+} binding in aLAC was initially less well defined. Even though both lysozymes

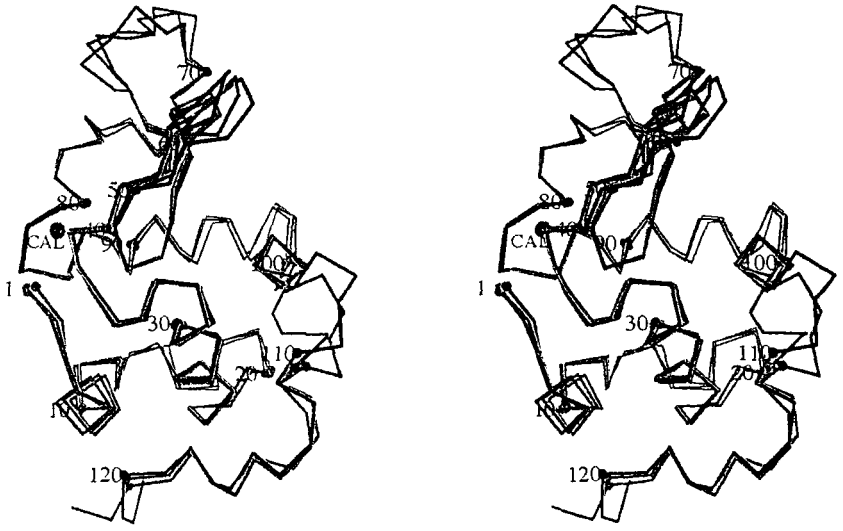


FIG. 20. Stereo view of an overlay of the structures of aLAC from human, guinea-pig, and goat. (From Pike *et al.* 1996 with permission.)

were shown to be Ca^{2+}-binding proteins (*261–263*) the crystal structure of the same proteins showed no electron density for Ca^{2+} (*264*). On the other hand a mutant of human LZ in which the two missing coordinating Asp residues were introduced showed both Ca^{2+} binding and a Ca^{2+} in the crystal, with a geometry analogous to that in human and baboon aLAC (*265*). In agreement with the Ca^{2+}-binding studies of LZ from horse and pigeon (*261–263*) a ^{43}Ca NMR study of Ca^{2+} binding to the two lysozymes and bovine and human aLAC showed very similar behavior, strong Ca^{2+} binding for one site, and the same symmetry of the site as revealed by the quadrupole coupling constant of the bound $^{43}Ca^{2+}$. Furthermore, the off rate of the bound Ca^{2+} was shown to be slow for all four proteins (*266*, *267*).

The Ca^{2+}-binding site in α-lactalbumin has some similarity with the EF-hand binding sites in that it consists of a helix–loop–helix motif, with all protein ligands contained in the loop. However, the pattern of ligands is not the same, and the site in aLAC has been named an "elbow" to differentiate it from the EF hand (*260*, *268*). Another difference between these two kinds of Ca^{2+}-binding sites is that the elbow is a single site whereas the EF hands appear in pairs (discussed earlier). Crystal structures of several aLACs are available (*260*, *269*, *270*) and are all very similar. The structure is divided into a large domain (α domain) and a small domain (β domain) by a cleft (Fig. 20) (*270*).

The Ca^{2+}-binding site is in the interface between the two domains. The only variation between the various structures occurs for residues 101–110. In the structure from human and recombinant bovine aLAC, residues 105–110 adopt a distorted α-helical conformation, whereas in goat and guinea-pig aLAC these residues form a loop (*270*). The difference may be due to different crystallization conditions and may indicate that this region is flexible in solution (*271*). Mutations in the flexible loop indicate that this is important for the interaction with GT.

Recently there have also appeared studies on the thermodynamics of the Ca^{2+}-loaded partially unfolded state (*272*), the importance of the disulfide bonds (*273*), and the functional role of calcium-binding residues (*274*). Furthermore, aLAC has been a model for protein folding because it forms a molten globule state during folding (*275*, *276*), which will not be discussed here.

F. Cadherins

The cadherins form a family of cell–cell adhesion receptors (*277*, *278*). They are transmembrane glycoproteins with typically five tandemly repeated extracellular domains (CAD repeats) (*279*, *280*). The adhesive action of cadherins depends on the presence of Ca^{2+} (*278*). A detailed study of Ca^{2+} binding to E-cadherin domains has shown that two CAD repeats are needed for Ca^{2+} binding (*281*). It was shown that the domain pair comprising the two N-terminal domains, ECAD12, bind Ca^{2+} strongly compared to the extracellular Ca^{2+} concentration, whereas individual domains showed no or very weak Ca^{2+} affinity. A crystal structure solved for the N-terminal domain of N cadherin shows that the polypeptide fold includes seven β-strands arranged in two β-sheets with the N and C termini at opposite ends of the domain, making a linear arrangement of the five domains possible. A metal-ion binding site occupied by an Yb^{3+} has been identified with two bidentate ligands from Glu11 and Glu69 (*282*). The solution structure of the N-terminal domain of E cadherin in the presence of Ca^{2+} has been determined by NMR. Even though the Ca^{2+} cannot be "seen" by NMR, Ca^{2+} ligands were inferred from Ca^{2+}-dependent chemical shift effects. In this way Glu11, Glu69, and Asp100 were suggested as ligands (*280*, *283*), in agreement with the crystal data (*282*) in which two water molecules could also be identified as ligands, still resulting in an incomplete coordination of the metal ion. Recently the crystal structure of the two N-terminal domains of E cadherin was solved (*284*). The general location of the Ca^{2+}-binding site suggested in the

earlier studies is confirmed; however, it is now shown that in each domain–domain interface there are three Ca^{2+}. Furthermore, the structure reveals a twofold symmetric dimer. The dimeric structure is in good agreement with solution state studies, which have shown a Ca^{2+}-dependent dimerization of the same protein fragment (*281*). A model based on the assumption that the relative orientation of the two domains in ECAD12 is also used for the other linking regions in a 240-Å-long extracellular region. This is in good agreement with a value of 220 Å obtained by electron microscopy (*285*) in the presence of calcium.

G. SPARC

The protein SPARC (*S*ecreted *P*rotein *A*cidic and *R*ich in *C*ysteine) is also known as BM-40 and osteocalcin (*286–288*). It was originally isolated from bone but is found in other matrix-producing tissues such as ligaments. It is also found in corticosteroid-secreting tissues such as the adrenal cortex; in epithelia in, for example, the gut and skin, and in platelets (*289*). It is a multidomain protein that is unique among extracellular proteins in that it possesses an EF-hand pair motif in the last of its three domains. The first two domains consist of a variable acidic domain and a follistatin-like module. It shares this domain organization with other proteins such as the brain protein SC1 (*290*) and the retinal protein QR1 (*291*). In addition to calcium, it has been shown to bind a number of extracellular proteins *in vitro,* including collagen, plasminogen, and albumin (*289*). It is believed to be a modulator of cell–matrix interactions.

The structure of the calcium-binding domain of SPARC was determined by X-ray crystallography to a resolution of 2.0 Å (*292*). The structure of the EF-hand pair resembled those found in intracellular proteins but had several unique features. In the first EF hand, there was a single residue insertion, which alters the fold of the calcium-binding loop such that a *cis*-peptide bond is formed and a backbone carbonyl acts as a calcium ligand. In the second EF hand, a disulfide bond is found, which may act to stabilize the calcium-binding loop. Finally, rather than having the hydrophobic interior of the EF-hand pair available for binding of target proteins, an amphiphilic amino-terminal helix binds in the cleft. The structure of the calcium-binding domain together with the second follistatin-like domain has also been determined (*293*).

The effect of calcium binding on the structure of SPARC has not been determined at molecular resolution. However, what is known

about the calcium binding is that the EF-hand pair contains one high-affinity and one low-affinity site (*294*). Calcium binding induces a conformational change resulting in an increase in helical structure as monitored by circular dichroism (*295*). Curiously, this apparent increase in regular structure does not alter the stability of the protein as measured by chemical denaturation.

V. Summary

In this review of calcium-binding proteins, we have not attempted to give an exhaustive summary of all calcium-binding proteins, but have considered some of the best examples, especially those for which high-resolution structural data are available. However, even this limited subset of the hundreds of calcium-binding proteins identified to date demonstrates the myriad ways in which nature uses calcium to regulate biological processes. It also demonstrates how finely tuned each protein is with respect to its environmental parameters such as calcium concentration as well as rates and magnitudes of changes in this concentration. It also shows the many ways in which the calcium-binding proteins are adapted to the particular target, whether it be a single protein, many proteins, membranes, or simply calcium itself. Despite the wealth of information available to us, much is left to learn about the functions and mechanisms of the calcium-binding proteins. Readers interested in further information are encouraged to explore the articles and reviews listed in the references.

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