Domain organization of calbindin D_{28k} as determined from the association of six synthetic EF-hand fragments

SARA LINSE,¹ EVA THULIN,¹ LIDA K. GIFFORD,² DINA RADZEWSKY,²
JAMES HAGAN,² ROSEMARIE R. WILK,³ AND KARIN S. ÅKERFELDT²

¹Physical Chemistry 2, Chemical Centre, University of Lund, S-221 00 Lund, Sweden

²Department of Chemistry, Rutgers University, Camden, New Jersey 08102

³DuPont Merck Pharmaceutical Co., Wilmington, Delaware 19880-0500

(RECEIVED February 24, 1997; ACCEPTED July 7, 1997)

Abstract

Calbindin D_{28k} is an intracellular Ca²⁺-binding protein containing six subdomains of EF-hand type. The number and identity of the globular domains within this protein have been elucidated using six synthetic peptide fragments, each corresponding to one EF-hand subdomain. All six peptides were mixed in equimolar amounts in the presence of 10 mM Ca²⁺ to allow for the reconstitution of domains. The mixture was compared to native calbindin D_{28k} and to the sum of the properties of the individual peptides using circular dichroism (CD), fluorescence, and ¹H NMR spectroscopy, as well as gel filtration and ion-exchange chromatography. It was anticipated that if the peptides associate to form native-like domains, the properties would be similar to those of the intact protein, whereas if they did not interact, they would be the same as the properties of the isolated peptides. The results show that the peptides in the mixture interact with one another. For example, the CD and fluorescence spectra for the mixture are very similar to those of the intact calbindin D_{28k}, suggesting that the mixed EF-hand fragments associate to form a native-like structure. To determine the number of domains and the subdomain composition of each domain in calbindin D_{28k}, a variety of peptide combinations containing two to five EF-hand fragments were studied. The spectral and chromatographic properties of all the mixtures containing less than six peptides were closer to the sum of the properties of the relevant individual peptides than to the mixture of the six peptides. The results strongly suggest that all six EF-hands are packed into one globular domain. The association of the peptide fragments is observed to drive the folding of the individual subdomains. For example, one of the fragments, EF2, which is largely unstructured in isolation even in the presence of high concentrations of Ca²⁺, is considerably more structured in the presence of the other peptides, as judged by CD difference spectroscopy. The CD data also suggest that the packing between the individual subdomains is specific.

Keywords: calbindin D_{28k}; Ca²⁺-binding protein; domain organization; EF-hand; protein folding; spectroscopy; subdomain association; synthetic peptides.

The EF-hand (Kretsinger & Nockolds, 1973) is a highly conserved helix-loop-helix structural motif present in a wide variety of calcium-binding proteins whose functions are to act as calcium buffers or to regulate specific target proteins in a calcium-dependent manner. To date, hundreds of EF-hands have been identified through sequence homology (Kretsinger 1987; Nakayama et al., 1992). One EF-hand is referred to as a subdomain. It is the packing arrangement of two or more EF-hand subdomains into a functional do-

main that confers the specific biological functions to the individual protein.

Many residues in the helix-loop-helix motif are highly conserved, particularly in the calcium-binding loop (Fig. 1). Although there exists a high degree of sequence homology among the EF-hands, they have been found to associate into different types of domain structures, typically containing between two and eight EF-hand subdomains associated into one or more globular domains. Due to interactions between the subdomains within a domain, the binding of Ca²⁺ typically occurs with positive cooperativity. The smallest type of domain consists of two EF-hands packed into a four-helix bundle. The Ca²⁺-binding loops, which are positioned at the same end of the bundle, form a short β -sheet. One domain of this type is present in calbindin D_{9k} (Szebenyi & Moffat, 1986;

Reprint requests to: Sara Linse; Physical Chemistry 2, Lund University, Chemical Centre, P.O. Box 124, S-221 00 Lund, Sweden; e-mail: sara@bor.fkem2.lth.se or to Karin S. Åkerfeldt; akersfeldt@crab.rutgers.edu.

Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; FPLC, fast protein liquid chromatography

Fig. 1. Amino acid sequences of calbindin D_{28k} and the synthetic peptide fragments, EF1–EF6. The EF-hand consensus sequence is shown in the middle and consensus residue positions are indicated by shading. In the consensus, J = hydrophobic residue.

Svensson et al., 1992). Calcyclin also contains a two-site arrangement; however, this protein naturally occurs as a homodimeric four-EF-hand entity (Potts et al., 1995).

In proteins containing more than two EF-hands, a number of domain arrangements have been observed. Parvalbumin contains three EF-hand sequences, two of which bind Ca²⁺. In this case, the EF-hands are associated into a trimer in which the third empty site acts as a lid to cover the hydrophobic surface generated by the other two subdomains (Kretsinger & Nickolds, 1973; Permyakov et al., 1991). Proteins containing four EF-hand subdomains may be arranged into two four-helix bundle domains connected with a linker, generally susceptible to proteolytic cleavage, as in calmodulin (Babu et al., 1988; Kretsinger et al., 1986; Finn et al., 1995; Kuboniwa et al., 1995; Zhang et al., 1995), troponin C (Herzberg & James, 1988; Satushyr et al., 1988; Gagné et al., 1995), and calcineurin (Griffith et al., 1995). In other proteins, four EF-hands pack into a single globular domain, as has been found in recoverin (Flaherty et al., 1993) and the sarcoplasmic Ca²⁺-binding protein (Vijay-Kumar & Cook, 1992; Cook et al., 1993). Presently, there are no known examples of domains containing more than four EF-hands,⁴ although there are a number of possible candidates, such as calretinin, calbindin D_{28k}, and reticulocalbin, all containing six subdomains, as well as LpS1 protein from sea urchins, which contains eight putative EF-hands.

It has been shown in several cases that the packing interactions among the EF-hands within a globular domain are strong and highly specific. A stoichiometric mixture of peptide fragments corresponding to the two EF-hands present in calbindin D_{9k} elute together and at the same salt concentration as the native protein by ion-exchange chromatography. (Finn et al., 1992). This example shows that, although the subdomains are no longer covalently connected, the interactions between the sites are strong and specific enough to remain intact even in the presence of high salt concentrations. NMR studies confirm that the two disconnected fragments come together to

form a native-like structure (Finn et al., 1992). Similarly, a peptide fragment, corresponding to one of the EF-hands of troponin C, was shown by NMR spectroscopy to exist as a homodimer in solution very similar in structure to the natural heterodimer (Shaw et al., 1992b). When this peptide was mixed stoichiometrically with its native EF-hand partner, it formed the heterodimer at the expense of the homodimer (Shaw et al., 1992a). Among the large domain types, parvalbumin has been reconstituted from two fragments containing one and two EF-hands, respectively (Permyakov et al., 1991), and the sarcoplasmic Ca²⁺-binding protein has been reconstituted from two fragments containing two EF-hands each (Durussel et al., 1993). These two EF-hand fragments exist as homodimers in isolation but, when they are mixed with each other, the native (heterodimeric) four-EF-hand domain is formed, observable as a shift in the tryptophan fluorescence spectrum from ca. 340 to around 330 nm (Durussel et al., 1993).

Based on the examples discussed above, it should, in principle, be possible to reconstitute and identify the domains present in a protein of unknown domain organization. The assumption is that, within a mixture of peptide fragments corresponding to the EF-hands of a particular protein, the subdomains strongly prefer to associate with their natural partners to form native-like domains as opposed to other non-native assemblies. In this work, the hypothesis was tested on one of the larger EF-hand proteins, calbindin D_{28k}, which contains six putative EF-hand motifs based on sequence homology to the canonical EF-hand (Fullmer & Wasserman, 1987). The three-dimensional structure for this protein is unknown. Calbindin D_{28k} is abundant in mammalian brain and sensory neurons. Mice lacking calbindin D_{28k} have recently been shown to suffer from impaired motor coordination due to altered neuronal Ca²⁺-homeostasis (Airaksinen et al., 1997). In previous work, six 33-residue peptides corresponding to the six EF-hand motifs of calbindin D_{28k} were synthesized and it was shown that five of them bind calcium as isolated peptides (Åkerfeldt et al., 1996). In the present study, the peptides have been mixed in stoichiometric amounts in different combinations to allow for the reconstitution of globular domains. The mixtures have been analyzed by CD, ¹H NMR, and fluorescence spectroscopy, as well as by gel filtration and ion-exchange chromatography.

Experimental strategy

EF-hand subdomains of calbindin D_{28k}

The EF-hand helix-loop-helix motif is a highly conserved 29-residue sequence (Kretsinger, 1987). The synthetic peptides corresponding to the six EF-hand sequences of calbindin D_{28k} contain 33 residues each, including two additional residues on each side of the consensus sequence, as shown in Figure 1. Cysteine residues occurring in the native sequence have been replaced by serine. The peptides are numbered EF1 through EF6 according to their position in the sequence starting from the N-terminus. EF1, EF3, EF4, and EF5 show good agreement with the consensus sequence whereas EF2 and EF6 appear to contain variant EF-hands. A set of Arabic numbers is used to specify the peptide mixture combinations, e.g., 123456 is the mixture of all six peptides and 156 is a mixture of EF1, EF5, and EF6.

Possible domain arrangements

There are many possible domain arrangements for calbindin D_{28k}, that contains six EF-hand subdomains. To name a few possibilities,

⁴Note added in proofs: The three-dimensional structure has now been presented for a five-EF-hand fragment of calpain which forms a homodimeric ten EF-hand domain (Blanchard et al., 1997; Lin et al., 1997).

the protein may consist of three domains of two EF-hands each, two domains of three each, two domains of a two-plus-four combination, one single domain of six sites, or one domain of five EF-hands in combination with a lone site. In proteins with multiple globular domains, each domain is formed by a contiguous segment of polypeptide (Creighton, 1993), and this rule is fulfilled by all EF-hand proteins with known three-dimensional structures. Thus, in testing different peptide combinations, we have predominantly evaluated mixtures of the type 123 and 456, rather than 145 and 236. We have also extended our studies to investigate some specific circular permutations, such as 561 (and 234), 126 (and 345) and 16 (and 2345), and all pentameric mixtures to probe the effect of losing one subdomain.

Domain reconstitution

The experiments are based on the assumption that the interactions between the EF-hands are specific enough that when all six peptides are mixed in equimolar amounts the peptides will associate with their natural partners to form the globular domains present in the intact protein. In the mixture 123456, each peptide was present at a concentration of 100 μ M to provide a total peptide concentration of 600 μ M. The same set of experiments were carried out for each individual peptide, as well as for intact calbindin D_{28k} . The native protein was studied at 100 μ M, and the individual peptides both at 100 μ M and 600 μ M. The properties of the mixture 123456 can thus be compared to those of calbindin D_{28k} and the sum of the individual peptides at 100 μ M each, or to the sum at 600 μ M each times a factor of 1/6 to correct for concentration effects. The same techniques were also used to investigate equimolar mixtures containing two to five peptides under the assumption that the properties of complementary domain mixtures will add up. For example, if the protein contains two domains, one comprising EF-hands 1–3 and the other EF-hands 4–6, the properties of the mixture of all six peptides will be the sum of the properties of the two mixtures 123 and 456. Thus, a study of many different mixtures should provide a guide to which EF-hand subdomains are present within each domain in the native protein.

Results

CD spectroscopy

In the presence of 10 mM Ca^{2+} , the CD spectrum of the mixture of all six peptides (123456) differs significantly from the sum of the spectra of the individual peptides (Fig. 2A). As indicated by the ellipticity at 222 nm, the sextet mixture is considerably more α -helical and the overall shape of the spectrum differs from the sum of the spectra of the individual peptides. The shape of the CD spectrum of 123456 is very similar to that observed for the intact protein (Fig. 2A).

The sextet is considerably more α -helical than the mixtures containing two to five fragments, as judged from the ellipticity at 222 nm. The spectra of the pentamer mixtures superimposed onto the spectrum of the sextet is shown in Figure 3A. In all of the pentamer spectra, the signal at 222 nm is considerably less intense than that of the sextet spectrum. In addition, none of the pentameric mixtures gives a CD spectrum with the same shape as that of 123456. The spectra of the pentamers differ slightly in intensity among each other but each one is close to the sum of the spectra of the constituent peptides. Similarly, the quartet (data not shown), triplet (Fig. 3B), and doublet mixtures (data not shown) give CD-spectra with shapes distinctly different from that of the

Fig. 2. CD spectra obtained in 2 mM Tris/HCl, pH 7.5, and in a 0.1-mm cuvette. **A:** An overlay of the following CD spectra obtained in the presence of 10 mM Ca^{2+} : 123456, containing 100 μ M of each peptide (—), 100 μ M calbindin D_{28k} (---), the sum of the spectra obtained for each individual peptide at 600 μ M times a factor of 1/6 (— · —) and the sum of the spectra obtained for each individual peptide at 100 μ M (· · · · ·). **B:** An overlay of the following spectra obtained in the absence of Ca^{2+} : 123456, containing 100 μ M of each peptide (—); 100 μ M calbindin D_{28k} with 1 mM EDTA added (---), and the sum of individual peptides at 100 μ M (· · · · ·).

sextet. These spectra are much closer to the sum of the constituent peptides, as exemplified in Figure 3B (for some of the triplet combinations).

The CD spectra of the individual peptides are concentration dependent. In the spectra discussed above, the individual peptide concentration was 100 μ M. To ensure that the observed differences between the mixtures and 123456 are not merely a concentration effect, some of the mixtures were recorded at a total peptide concentration of 600 μ M. Spectra of dimeric, trimeric, and pentameric mixtures were thus recorded at individual peptide concentrations of 300, 200, and 120 μ M, respectively. In each case the

Fig. 3. CD spectra obtained in a 0.1-mm cuvette and in 2 mM Tris/HCl, pH 7.5, containing 10 mM Ca^{2+} and 100 μM of each constituent peptide. **A:** A comparison of the CD spectra obtained for 123456 (—), and the pentet mixtures 12345 (—), 12346 (---), 12356 (—), 12456 (---), 13456 (—), and 23456 (—). **B:** The superimposed CD spectra obtained for the triplets, 456 (—) and 126 (---), the sum of individual peptides, 4 + 5 + 6 (---) and 1 + 2 + 6 (—), and the sextet, 123456 (—).

shape of the spectrum was different from that obtained for 123456. Also, in comparing the spectra of the mixtures to the sum of their individual peptide constituents, the largest differences are observed for 123456 relative to 1 + 2 + 3 + 4 + 5 + 6.

The experiment was repeated in Ca^{2+} -free buffer. The CD spectrum obtained for the mixture of the six peptides in the absence of Ca^{2+} deviates considerably from that of apo calbindin $\text{D}_{28\text{k}}$ (Fig. 2B). The spectrum of the mixture also deviates from the sum of the individual peptides, although there is no significant gain in the signal at 222 nm.

^1H NMR spectroscopy

The one-dimensional ^1H NMR spectrum of the mixture containing an equimolar amount of all six peptides was acquired in water in the presence of 10 mM Ca^{2+} (Fig. 4). In this spectrum, no narrow resonances are observed, as would be expected if the peptides were unassociated; instead, the line widths are relatively broad and comparable to those observed for intact calbindin $\text{D}_{28\text{k}}$. Broad line widths could be due to the self-association of the individual peptides. As has been shown in earlier work (Åkerfeldt et al., 1996), broader line widths are indeed obtained in the spectra of EF1, EF6, and particularly EF3, at equivalent concentrations; consistent with that, these peptides self-associate to form higher order oligomers. Narrow line widths are observed in the spectra of EF2, EF4, and EF5, both at 100 and 600 μM , suggesting that these remain unassociated or form lower order oligomers. When the spectra of the individual peptides are superimposed (Fig. 4), it is clear that the line widths are considerably more narrow than in the spectrum of 123456. Calbindin $\text{D}_{28\text{k}}$ has a larger number of resonances around 7 ppm, but a direct chemical shift comparison cannot be made between the native protein and 123456 because the latter lacks as many as five aromatic residues present in the segments linking the EF-hands in the native protein.

Fluorescence spectroscopy

The fluorescence spectra, acquired in the presence of 10 mM Ca^{2+} , are dominated by contributions from EF1 and EF3, each containing one tryptophan residue. In the individual spectra, EF1 and EF3 show fluorescence emission maxima at 341 and 340 nm, respectively. The sum of the spectra recorded for each of the six peptides is centred around 339 nm. In the spectrum of 123456, the maximum has blueshifted to 329 nm, which is at the same wavelength where it appears for calbindin $\text{D}_{28\text{k}}$, although the peak for the native protein is somewhat more narrow (Fig. 5A). The mixture 123 gives a peak centered about 339 nm, and the spectral sum of the two combinations, 123 and 456, is almost identical to the sum of the individual peptides. However, in the sum of the pairwise mixtures, 12, 34, and 56, the fluorescence maximum appears approximately halfway between those of the sum of the individual peptides and the mixture, 123456 (Fig. 5B), primarily because the fluorescence of the tryptophan in EF3 at 340 nm blueshifts to 334 nm when mixed with EF4. In contrast, the spectrum of EF1 yields a maximum at 341 nm, which is similar to that observed for the pair 12 (339 nm). The spectra recorded for 234, 345, and 2345 are all very similar to that of 34.

Size exclusion chromatography

In the presence of calcium, the mixture 123456 elutes as one peak on a Superdex75 gel filtration column. The front of the peak is very sharp, whereas the back side declines slowly. As judged by MALDI-TOF mass spectrometry and ion-exchange chromatography, the front of the peak contains all six peptides. When individually injected on the gel filtration column, all six peptides elute at a later retention times with the exception of EF5, which elutes at a similar retention time as the mixture 123456.

Ion-exchange chromatography

When injected one by one on the ion exchange column with 10 mM Ca^{2+} , each peptide exhibits a distinct retention time

Fig. 4. ^1H NMR spectra obtained at 500 MHz in $\text{H}_2\text{O}:\text{D}_2\text{O}$ 9:1, pH 7.0, at 27°C , in the presence of 10 mM Ca^{2+} , for calbindin D_{28k} at 100 μM (top) and 123456, containing 100 μM of each peptide (middle). The bottom panels show the sum of the spectra of the six peptides, recorded individually at 600 μM (EF2, EF3, EF4, and EF5) or 100 μM (EF1 and EF6). The spectra were weighted before the sum was calculated. The A panels show the spectral region for the aromatic and amide protons (6–11 ppm) and the B panels show the region for the aliphatic protons (–1 to 4 ppm).

(Fig. 6, bottom panel). The EF6 peptide, which is not retained on the column due to its zero net charge (or +2 if stoichiometrically complexed with Ca^{2+}), is followed by EF2, –3 (–1); EF4, –4 (–2); EF3 –4 (–2); EF1 –5 (–3); and EF5 –6 (–4). The elution volumes are consistent with the number of charges except in the cases of EF4 and EF3, which are separated although they carry the same net charge.⁵

Ion-exchange chromatography of a stoichiometric amount of all six EF-hand peptides, 123456, in the presence of 10 mM calcium provides six separate peaks with positions differing from those of the individually injected peptides (Fig. 6). Each peak corresponds to one of the six peptides, as confirmed by mass spectroscopy of the isolated peaks. When mixed together, the peptides are, with the exceptions of EF6 and EF5, retained longer on the column compared to when they are run individually, which is a strong indication that the peptides in the mixture interact with each other.

To test if the EF-hand domains in the native protein are arranged linearly in a combination of three domains of two EF-hands each or as a combination of two domains of three EF-hands each, the

peptide mixtures 12, 34, and 56, as well as 123 and 456, were tested (Fig. 6). In these cases, the observed retention times are similar to those of the individually injected peptides, with the exception of 456, in which EF4 has an elution volume intermediate between that of EF4 alone and EF4 in 123456.

Ion-exchange chromatography was also performed on a variety of other mixtures containing two to five peptides. All possible pentet combinations (23456, 13456, 12456, 12356, 12346, and 12345) were tested. The peptides in these mixtures are retained longer on the column than when individually injected, but to a lesser extent than in the case of the 123456 mixture. The pentets 12346 and 23456 represent two extreme cases. In 12346, each peptide elutes similarly to the individual peptides, whereas in the pentet 23456, all peptides except EF3 are retained as much as in the mixture 123456. In the case of the quartet (1234, 3456, and 2345) and triplet combinations (234, 345, 126, 156, and 235), the effects on the retention times are relatively small. The exception is the triplet 234, for which the effects on elution volumes are significant but not as large as in 123456. The doublet combinations 23, 34, 45, 35, 25, and 24 were also tested. In each case, the retention time is unaffected by the presence of the other peptide except in the pairs 23 and 25 in which EF2 is slightly more retained than EF2 when injected by itself.

EF5 and EF6 elute at the same volume regardless of the mixture combination. Since EF6 has no intrinsic negative net charge, it

⁵Broad line widths are observed in the ^1H NMR spectrum of EF3, which could be due to the formation of higher order aggregates at the peptide concentration used in the ion exchange experiment (Åkerfeldt et al., 1996). This may explain why EF3 elutes later than EF4. Factors other than charge may also affect the retention time.

Fig. 5. Fluorescence emission spectra obtained in 2 mM Tris/HCl, pH 7.5, in the presence of 10 mM Ca^{2+} . **A:** The superimposed spectra of 123456, containing 100 μM of each peptide, (—), calbindin $\text{D}_{28\text{k}}$ at 100 μM (---), and the sum of the spectra obtained for the individual peptides 1 + 2 + 3 + 4 + 5 + 6 at 100 μM each (···). **B:** A comparison of the spectrum of 123456 (—) with the sum of spectra for the three pairs 12 + 34 + 56 (---) and the sum of the spectra of the two triplets 123 + 456 (···), in all cases with 100 μM of each peptide.

does not have a tendency to stick to the positively charged ion exchange column and elutes with the solvent front both when injected alone and together with other peptides. The retention time of EF5 is in all cases the same as for the individual peptide. We interpret this behavior to be an effect of EF5 having the highest intrinsic negative net charge. Since EF5 elutes considerably later than the other peptides, it has considerably more time to readjust to its individual retention time compared to the other peptides. Therefore, information on EF5 and EF6 can only be gained indirectly by comparing mixtures that contain these peptides to those that lack them, for example, by comparing 123456 to 12346 and 126 to 12. The peptides exhibiting a change in elution volume are EF2, EF3, EF4, and, to a lesser extent, EF1. A compilation of their retention times in the different peptide combinations can be seen in Figure 7.

For comparison, the ion-exchange chromatography experiment was repeated using calbindin $\text{D}_{9\text{k}}$ and two fragments, each containing one EF-hand subdomain. As shown in Figure 8, a mixture of the two fragments run distinctly different from each individual fragment. Instead of eluting as two separate peaks, the two fragments appear to form a stoichiometric complex that elute at the

Fig. 6. Ion-exchange chromatography profiles. At the bottom of the panel is shown an overlay of the six chromatograms obtained for the six peptides injected individually at 100 μM each. Solid lines are used for EF1, EF4, and EF6, and dashed lines for EF2, EF3, and EF5. The top of the panel contains the chromatogram of calbindin $\text{D}_{28\text{k}}$. The second trace shows the elution profile of the mixture 123456, with 100 μM of each peptide, followed by 12, 34, 56, 123, and 456, as indicated to the right of each chromatogram. The identity of each peak, as confirmed by mass spectroscopy, is indicated by the number of the corresponding EF-hand: 1 for EF1, 2 for EF2, and so on.

same volume as the intact protein. Similar results have previously been reported using agarose gel electrophoresis (Finn et al., 1992).

Discussion

Interactions between subdomains

Five methods were used to assay the ability of the EF-hand peptides to associate with their natural partners in the presence of Ca^{2+} to form the domains present in calbindin $\text{D}_{28\text{k}}$. Structural changes induced by the interactions between the subdomains were monitored by CD, fluorescence, and NMR spectroscopy, while the

Fig. 7. A compilation of the ion-exchange chromatography elution volumes for EF1, EF2, EF3, and EF4 when injected alone (bottom of each panel) or in different mixtures, as indicated on each bar.

Fig. 8. Ion-exchange chromatography profiles of P43M, (the Pro43 → Met mutant form of calbindin D_{9k}), the two EF-hand fragments f1 and f2 (generated from P43M by CNBr-cleavage), as well as an equimolar mixture of f1 and f2. Fragments f1 and f2 span residues 1–43 and 44–75, respectively. Thus, f1 includes the N-terminal and f2 the C-terminal EF-hand subdomain of calbindin D_{9k} (Finn et al., 1992). The concentration of injected P43M was 100 μ M and of f1 and f2 100 μ M each.

results from CD spectroscopy and ion-exchange chromatography provide additional insights into the specificity of the interactions among the peptides.

The results from all three spectroscopic techniques give strong indications that all of the peptides interact with one another when mixed. The shape of the CD spectrum of 123456 is very similar to that of native calbindin D_{28k} , but distinctly different from the sum of the spectra obtained for the individual peptides (Fig. 2A, B). The ^1H NMR spectrum of the mixture of all six peptides is, in a qualitative sense, more similar to the spectrum of native calbindin D_{28k} than to the sum of the spectra of the individual peptides (Fig. 4). The line widths in the spectrum of 123456 are considerably broader than those observed in the individual spectra of EF2, EF4, EF5, and EF6, suggesting extensive interactions among the peptides. In the sum of the fluorescence emission spectra obtained for each individual EF-hand peptide, the maximum is centered around 339 nm. In the spectra of intact calbindin D_{28k} and the mixture 123456 the maximum has blueshifted by ca. 10 nm to 329 nm, indicating that the tryptophan residues in EF1 and EF3 are in a similar dielectric environment in 123456 as in the native protein. A blueshift of similar magnitude was reported to occur on the reconstitution of the four-EF-hand domain in the sarcoplasmic calcium-binding protein (Durussel et al., 1993).

The gel filtration results corroborate earlier findings that some of the isolated peptides form homodimers or higher order oligomers; however, a stoichiometric mixture, 123456, elutes at a reduced volume, indicating the formation of larger molecular assemblies. The data show that homotropic complexes break up in favor of the formation of heterotropic complexes, implying that interactions between the natural partners are stronger than those between identical subdomains. The ion-exchange chromatography results also imply-

ing that the peptides interact with one another when mixed prior to injection. Although the number of peaks are the same as the number of mixed peptides, the elution volumes differ from those of the individually injected peptides. The CD data do not indicate that the peptides interact or associate in the absence of Ca^{2+} to form a structure similar to apo-calbindin $\text{D}_{28\text{k}}$.

Domain organization

To examine the domain arrangement of calbindin $\text{D}_{28\text{k}}$, the chromatographic and spectroscopic properties of the mixture 123456 were compared to those of the native protein. It was anticipated that the peptides in the mixture would associate to yield the native domains present in the protein. Mixtures containing less than six EF-hand peptides were analyzed and their properties compared to those of 123456. The sum of the properties were generated for each set of mixtures and compared to the properties of 123456. For example, the sum of the CD spectra of the two mixtures 123 and 456, or of 1234 plus 56, were compared to the spectrum of 123456.

If calbindin $\text{D}_{28\text{k}}$ consists of two domains of 123 plus 456, for example, one may expect that the sum of the CD or fluorescence spectra obtained for these two mixtures would closely match those of 123456. In addition, the retention times of the peptides in the mixtures 123 and 456 would be similar to those in 123456. In contrast, the sum of peptide mixtures that do not correspond to domains would be closer to the sum of the individually studied peptides.

The CD spectra of the mixtures containing two to five peptides were, in all cases, very similar to the sum of the spectra of the corresponding single peptides. In other words, all six EF-hands need to be present to generate a CD-spectrum similar to that of native calbindin $\text{D}_{28\text{k}}$, suggesting that they are all part of a single domain. For example, the sum of the spectra of the dimers 12 + 34 + 56 is very similar to the sum of the spectra of the individual peptides and significantly different from the spectrum of 123456 (Fig. 9A). A comparable result is obtained by taking the sum of other combinations of dimers, such as 16 + 23 + 45 (Fig. 9A). Likewise, it is not possible to regain the spectrum of 123456 by taking the sum of two trimer spectra. The sums of the triplet

Fig. 9. The CD spectrum of the sextet 123456 (—) compared to different sums of spectra. **A:** The sum of three pairs: 12 + 34 + 56 (—) and 23 + 45 + 16 (---). **B:** The sum of triplet combinations: 123 + 456 (—), 156 + 234 (---), and 126 + 345 (- · -). **C:** The sum of a quartet and a pair combination: 1234 + 56 (—) and 12 + 3456 (---). **D:** The sum of a pentet and singlet combination: 12456 + 3 (—), 13456 + 2 (---), and 23456 + 1 (- · -). All the spectra were recorded in a 0.1-mm cuvette in 2 mM Tris/HCl, pH 7.5, and in the presence of 10 mM Ca^{2+} .

combinations, 123 + 456, 234 + 156, or 345 + 126, are closer to the sum of individual peptides than to the sextet (Fig. 9B). Thus, the CD data argue against the presence of discrete globular domains the size of two or three EF-hands. Similarly, there is no evidence for an arrangement with a four-plus-two (Fig. 9C) or a five-plus-one (Fig. 9D) domain organization.

The fluorescence data are inconsistent with an arrangement with two three EF-hand domains as the spectral sum of 123 plus 456 is similar to the sum of the individual peptides. However, the fluorescence maximum in the spectral sum of 12 + 34 + 56 falls between those of 123456 and the sum of the spectra obtained for the single peptides. This mainly reflects the shift in the fluorescence maximum obtained when EF3 is mixed with EF4, suggesting significant interactions between these two peptides, although the environment of EF3 does not seem to be as close to native as when the remaining four peptides are added.

There is no combination in which all the peptides are retained as strongly on the ion exchange column as with 123456, implying that they are all part of a single domain. When the calbindin D_{28k} peptides are injected as pairs (Fig. 6), their retention times are only slightly different from those of the individual peptides, in marked contrast to calbindin D_{9k} (Fig. 8). If calbindin D_{28k} exists as three two-site domains, it would also be expected that a stoichiometric mixture of all six EF-hand peptides, 123456, would elute as three peaks, containing two peptides each, as seen in the case of calbindin D_{9k} . Likewise, the mixture 123 yields three peaks with very similar retention times to those of the individual peptides (Fig. 6), which argues against two three-site domains arranged linearly in sequence.

Specificity of subdomain packing

When compared to the sextet, the CD spectra of the pentet mixtures are indicative of specificity in the aggregate formed, since none of the pentet spectra has the same shape as the sextet spectrum. This suggests that the peptides cannot substitute for one another at random in the complex to regain a native-like structure. If the interactions were not specific, one would expect that another peptide in the pentet mixture could take the place of the missing peptide to form a complex with a structure similar to the complex formed in 123456. If each EF-hand peptide could take the position of any other subdomain, one would expect that the mean residue ellipticity of some pentet combination would be as high as that observed for 123456. In the mixture 12345, for example, the complexes would then consist of 20% 112345, 20% 122345, 20% 123345, 20% 123445, and 20% 123455.

Association-induced folding of subdomains

The CD spectra provide evidence for a coupling between the association and folding of the EF-hand peptides. An alternative approach to assessing the structural differences between the pentet and sextet mixtures is to analyze the difference spectrum obtained by subtracting the pentet from the sextet and comparing the resulting spectrum to that of the missing peptide. In each of the six cases, the spectral difference is not equivalent to the spectrum of the missing peptide at 100 μ M nor at 600 μ M multiplied by a factor of 1/6, as shown in Figure 10. As judged from the ellipticity at 222 nm, there is more α -helical structure present in the difference spectrum obtained by subtracting any of the pentet mixtures from 123456 than in the isolated "missing" peptide. This suggests

a more structured complex when all six peptides are present. A striking example is shown in the difference spectrum obtained by subtracting 13456 from 123456 and comparing it to the spectrum of EF2. EF2, which is largely unstructured as an isolated peptide, is relatively structured in the presence of the other five peptides. The other peptides seem to provide important contacts that induce structure in EF2, a result that is indicative of an association-induced folding. The spectral change could also arise from the particular peptide increasing the helical content of the other five subdomains. This may not be the case for EF2, which is unfolded on its own, but is likely to occur for peptides with spectra indicative of a significant amount of structure, such as EF5 and EF3. The difference spectra for these subdomains still point to a net gain in α -helical structure on association with the other peptides, implicating an association-driven folding into a more stable complex.

Roles of particular subdomains

Each one of the peptides (EF1–EF6) appears to interact strongly with several subdomains in the complex. This is observed both directly and indirectly for EF2, EF3, and EF4, which are most strongly retained in 123456, but also significantly retained in many other mixtures. The pentet that lacks EF2, 13456, shows drastic differences in the retention times for EF3 and EF4 when compared to 123456. Important interactions between EF2 and EF3 and between EF2 and EF4 are also apparent when comparing the retention times of EF3 and EF4 in 23456 to 3456. Of the triplets studied, EF2 is most retained in 234 and 126. It is also retarded in the pairs 23 and 25. EF3 appears to interact with several other peptides since it is significantly more retarded in 123456 than in any other mixture studied. Similarly, the elution volume of EF4 is increased in 345, 234, and 456. EF5 appears to play a central role since the elution volumes of the peptides in the pentet lacking EF5, 12346, are close to those of the singly injected peptides. The importance of EF6 to the stability of the complex is seen indirectly when comparing the peptide retention times between mixtures that contain EF6 to those that do not (e.g., 123456 to 12345, 23456 to 2345, 126 to 12, and 456 to 45). EF1 appears to be less important than the central subdomains EF2–EF5. For example, adding EF1 to 23456 affects the retention time of EF3 only. On the other hand, adding EF1 to 3456 affects both EF3 and EF4. When pentamer mixtures are compared to 123456, it is clear that each one of EF2–EF6 has a large effect on the elution volume of EF1. For example, there is a large effect on the retention time of EF1 when adding EF6 to 12345.

The fluorescence spectroscopy data corroborate the ion exchange results in that they point to important interactions between the peptides EF3 and EF4 in the pair 34 (Fig. 5), but not between EF1 and EF2 in 12, or within 123.

Role of linker segments

It is interesting to compare the length of the segments connecting the subdomains among different EF-hand proteins. In calbindin D_{28k} , the linkers are relatively long and encompass 12 to 16 amino acid residues (counting 29 amino acids as the motif). Linkers of similar length are found in recoverin and the sarcoplasmic calcium binding protein, both of which contain large globular domains of four EF-hands. Shorter linkers of seven to nine amino acid residues are found in calbindin D_{9k} , calmodulin, and troponin C, which contain small domains of two EF-hands each. In the present study,

Fig. 10. CD difference spectra. In each panel is shown the difference spectrum obtained by subtracting the spectrum of the pentet from that of the sextet (—) compared to the spectrum of the single peptide that is missing in the particular pentet mixture (---). The concentration was 100 μ M of each peptide.

it was shown that the EF-hands in calbindin D_{28k} , like the subdomains in calbindin D_{9k} and troponin C, can associate with their domain partners even in the absence of most of the intervening linker segments. Thus, the message for subdomain association lies within the EF-hand motif itself and not in the connecting linker sequences.

Concluding remarks

The results of the present attempt to assess the domains in calbindin D_{28k} by reconstructing the protein from its EF-hand building blocks was a surprise in that the protein does not seem to contain structurally independent domains the size of two, three,

four, or even five EF-hands. Instead, the combined data, using five different methods, all agree and strongly suggest that the six EF-hands interact extensively with one another and that they are part of one large globular domain.⁶ Interactions between non-identical subdomains are preferred over self-association into homodimers or higher order oligomers. The packing among the peptides in the complex is specific and all six sites are needed to obtain a native-like structure.

This work represents a novel approach for elucidating the domain arrangement of an EF-hand protein, which we anticipate will be useful in determining the domains present in other proteins, as well as understanding the coupling between folding and association of subdomains into functional domains. The strategy would be of particular aid in investigating the domain organization of other large members of the EF-hand family for which little data is yet available. A better understanding of their functional and structural properties will, without doubt, contribute significantly to our current understanding of the biological roles of EF-hand proteins, as well as to identify what factors are responsible for making certain proteins act as buffers and others to serve regulatory functions. The knowledge of the domain organization is an important step in the functional characterization of an EF-hand containing protein, as has previously been demonstrated for calmodulin and troponin C, both of which contain two domains of two EF-hands each. Studies of their individual domains, obtained through proteolytic digest, have greatly facilitated the analysis of denaturation data (Brzeska et al., 1983), the assignment of strong versus weak binding sites for Ca^{2+} and other cations (Andersson et al., 1983; Martin et al., 1985; 1986), and the assessment of the cooperativity of Ca^{2+} -binding within each pair of sites. (Linse et al., 1991; Li et al., 1994). The knowledge of the domain organization could also be an important step toward the structural elucidation of an EF-hand containing protein. The three-dimensional structure is, so far, lacking for most of the members of the EF-hand superfamily and, to our knowledge, for all proteins containing more than four EF-hands (see footnote 5 on page 2386). However, these proteins appear to consist almost exclusively of EF-hand subdomains arranged into different types of domains. The identification of the domains present in the larger EF-hand proteins may direct efforts at solving the three-dimensional structure of the individual domains. With the growing number of high-resolution structures, it is also conceivable that patterns of different types of domain arrangements will emerge that can be used as a data base in modeling unknown structures by homology.

Materials and methods

Peptide synthesis and purification

The synthesis of the six 33-mer peptides by solid-phase methodology and purification by ion exchange and reversed-phase HPLC were recently described (Åkerfeldt et al., 1996).

Peptide solutions and buffers

The freeze-dried purified peptides were dissolved in water and the pH was adjusted to 7 with 0.1 M NaOH. The concentration of the

individual peptide solutions was determined by amino acid analysis following acid hydrolysis. The concentrations ranged from 0.4 to 1.1 mM. These peptide stock solutions were employed in the preparation of all mixtures described in this study. The peptides were mixed in equimolar amounts in different combinations, as described below. In the CD and ion-exchange chromatography experiments, the mixtures contained 100 μM of each constituent peptide and 10 mM Ca^{2+} in 2 mM Tris/HCl buffer, pH 7.5, unless otherwise stated. In a previous study it was shown that 2 mM Ca^{2+} was sufficient to saturate EF1, EF3, EF4, and EF5, while EF6 required higher Ca^{2+} -concentrations (Åkerfeldt et al., 1996). The solutions were made by adding the appropriate volumes of water and concentrated buffer stock solution. The NMR experiments were performed under identical conditions, except water was used instead of buffer, and the pH of the solution was adjusted to 7.0 with 0.2 M NaOH.

CD spectroscopy

CD spectra of single peptides and different peptide mixtures were obtained by averaging three to eight scans between 200–250 nm on a JASCO-720 spectropolarimeter at 25 °C in a thermostated 0.1 mm cuvette with the individual peptide concentrations kept at 100 or 600 μM . A baseline with pure buffer was recorded separately and subtracted from each spectrum. The spectra of intact calbindin D_{28k} , the sextet, and all the pentet mixtures were recorded twice using freshly prepared solutions, with high reproducibility. Spectra of all other mixtures were recorded once.

CD spectra of native calbindin D_{28k} (100 μM) and the following peptide mixtures were recorded with 100 μM of each peptide in 2 mM Tris/HCl, pH 7.5, containing 10 mM Ca^{2+} : one sextet (123456), six pentets (12345, 23456, 13456, 12456, 12356, and 12346), three quartets (1234, 3456 and 2345), six triplets (123, 126, 156, 234, 345 and 456), and six doublets (12, 16, 23, 34, 45 and 56). CD spectra were obtained for the individual peptides at both 100 μM and 600 μM . Spectra of the mixture 123456 and calbindin D_{28k} were also taken at 100 mM Ca^{2+} in the same buffer. In addition, spectra of the single peptides (100 μM), the mixture 123456 (100 μM of each peptide), and calbindin D_{28k} (100 μM) were recorded in the absence of Ca^{2+} . CD spectra of the following mixtures were also recorded: 45 (300 μM of each peptide), 234 (200 μM each) and 12345 (120 μM each).

^1H NMR spectroscopy

One-dimensional ^1H NMR spectra were recorded at 500 MHz on a GE-Omega spectrometer in H_2O (10% D_2O) at pH 7.0 and 25 °C using sample volumes of 250 μL in a Shigemi tube (Shigemi Co., Ltd., Tokyo). The mixture of all six peptides, 123456, was recorded at a concentration of 100 μM of each peptide. The spectrum of calbindin D_{28k} was recorded for a 100 μM solution, and those for single peptides at both 100 and 600 μM .

Fluorescence spectroscopy

Fluorescence spectra were recorded on a SPEX Fluorolog spectrometer using a 3×3 mm cuvette (45 μL sample volume). Both the excitation and emission bandwidth was 3 nm. Emission spectra between 290 and 430 nm (stepsize 1 nm) were recorded with the excitation set at 280 nm. The individual peptides were studied at 100 as well as 600 μM . The spectrum of the mixture 123456 was

⁶This conclusion is supported by recent results (Åkerfeldt, Thulin & Linse, unpubl. result) showing that a mixture of EF1, EF2 and a 4-EF-hand fragment comprising EF-hands 3–6 elute as one peak on the ion-exchange column in the presence of calcium. This peak elutes later than each of the three fragments and at a similar position to calbindin D_{28k} .

recorded with a peptide concentration of 100 μ M each and the intact protein was studied at 100 μ M.

Gel filtration

Gel filtration chromatography was accomplished on an analytical Superdex75 column (fractionation range 3–30 kDa; 3×300 mm; Pharmacia, Uppsala, Sweden), eluting with a buffer of 2 mM Tris/HCl, pH 7.5, containing 10 mM CaCl_2 and 150 mM KCl, using a Pharmacia FPLC system equipped with an LKB variable wavelength detector set at 214 nm.

Ion-exchange chromatography

Anion-exchange chromatography was accomplished on an analytical Mono-Q HR 5/5 column using a Pharmacia FPLC system equipped with an LKB variable wavelength detector set at 214 nm. The peptides were eluted with a gradient of 0–0.3 M NaCl in 2 mM Tris/HCl and 10 mM CaCl_2 over 30 min at pH 7.5 with a flow rate of 1 mL/min. In these experiments, a change in elution volume of ± 0.5 mL is within experimental error.

Acknowledgments

We are very grateful to Dr. Jannette Carey and to Dr. Robert Fairman for many stimulating discussions and their critical reading of the manuscript. This work was supported by the Swedish Natural Science Research Foundation (grant K 10178-305), the Wenner-Gren Foundations, the Research Corporation (grant CC4027), and the Research Council of Rutgers University.

References

- Airaksinen MS, Eilers J, Garaschuk O, Thoënen H, Konnerth A, Meyer M. 1997. Ataxia and altered dendritic calcium signalling in mice carrying a targeted null mutation of the calbindin D_{28k} gene. *Proc Natl Acad Sci USA*. Forthcoming.
- Andersson A, Forsén S, Thulin E, Vogel HJ. 1983. Cadmium-113 nuclear magnetic resonance studies of proteolytic fragments of calmodulin: Assignment of strong and weak cation binding sites. *Biochemistry* 22:2309–2313.
- Akerfeldt KS, Coyne AN, Wilk RR, Thulin E, Linse S. 1996. Ca^{2+} -binding stoichiometry of calbindin D_{28k} as assessed by spectroscopic analyses of synthetic peptide fragments. *Biochemistry* 35:3662–3669.
- Babu YS, Bugg CE, Cook WJ. 1988. Three-dimensional structure of calmodulin refined at 2.2 Å resolution. *J Mol Biol* 204:191–204.
- Blanchard B, Grochulski P, Li Y, Arthur SC, Davies PL, Elce JS, Cygle M. 1997. Structure of a calpain Ca^{2+} -binding domain reveals a novel EF-hand and Ca^{2+} -induced conformational changes. *Nature Struct Biol* 4:532–538.
- Brzeska H, Venyaminov SV, Grabarek Z, Drabikowski W. 1983. Comparative studies on thermostability of calmodulin, skeletal muscle troponin C and their tryptic fragments. *FEBS Lett* 153:169–173.
- Cook WJ, Jeffrey LC, Cox JA, Vijay-Kumar S. 1993. Structure of a sarcoplasmic calcium-binding protein from *Amphioxus* refined at 2.4 Å resolution. *J Mol Biol* 229:461–471.
- Creighton TE. 1993. *Proteins. Structure and molecular properties*. New York: W.H. Freeman and Company. p 254.
- Durussel I, Luan-Rilliet Y, Petrova T, Takagi T, Cox JA. 1993. Cation Binding and conformation of tryptic fragments of *Nereis* sarcoplasmic calcium-binding protein: Calcium-induced homo- and heterodimerisation. *Biochemistry* 32:2334–2400.
- Finn BE, Evenäs J, Drakenberg T, Waltho JP, Thulin E, Forsén S. 1995. Calcium-induced structural changes and domain autonomy in calmodulin. *Nature Struct Biol* 2:777–783.
- Finn BE, Kördel J, Thulin E, Sellers P, Forsén S. 1992. Dissection of calbindin D_{9k} into two subdomains by a combination of mutagenesis and chemical cleavage. *FEBS Lett* 298:211–214.
- Flaherty KM, Zozulya S, Stryer L, McKay DB. 1993. Three-dimensional structure of recoverin, a calcium sensor in vision. *Cell* 75:709–716.
- Fullmer CS, Wasserman RH. 1987. Chicken intestinal 28-kilodalton calbindin-D: Complete amino acid sequence and structural considerations. *Proc Natl Acad Sci USA* 84:4772–4776.
- Gagné SM, Tsuda S, Li MX, Smillie L, Sykes BD. 1995. Structures of the apo and calcium troponin-C regulatory domains: the muscle contraction switch. *Nature Struct Biol* 2:784–789.
- Griffith JP, Kim JL, Kim EE, Sintchak MD, Thomson JA, Fitzgibbon MJ, Fleming MA, Caron PR, Hsiao K, Navia MA. 1995. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell* 82:507–522.
- Herzberg O, James MNG. 1988. Refined crystal structure of troponin C from turkey skeletal muscle at 2.0 Å resolution. *J Mol Biol* 203:761–779.
- Kretsinger RH. 1987. Calcium coordination and the calmodulin fold: Divergent versus convergent evolution. In: *Cold Spring Harbor Symposia on Quantitative Biology, Vol. LII*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, pp 499–510.
- Kretsinger RH, Nockolds CEJ. 1973. Carp muscle calcium-binding protein. *J Biol Chem* 248:3313–3326.
- Kretsinger RH, Rudnick SE, Weissman LJ. 1986. Crystal structure of calmodulin. *J Inorg Biochem* 28:289–302.
- Kuboniwa H, Tjandra N, Grzesiek S, Ren H, Klee CB, Bax A. 1995. Solution structure of calcium-free calmodulin. *Nature Struct Biol* 2:768–776.
- Li MX, Chandra M, Pearlstone JR, Racher KI, Trigo-Gonzales G, Borgford T, Kay CM, Smillie LB. 1994. Properties of isolated recombinant N and C domains of chicken troponin C. *Biochemistry* 33:917–925.
- Lin G, Chattopadhyay D, Maki M, Wang KKW, Carson M, Jin L, Yuen P, Takano E, Hatanaka M, DeLucas LJ, Narayana SVL. 1997. Crystal structure of calcium bound domain VI of calpain at 1.9 Å resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nature Struct Biol* 4:539–547.
- Linse S, Helmersson A, Forsén S. 1991. Calcium binding to calmodulin and its globular domains. *J Biol Chem* 266:8050–8054.
- Martin SR, Andersson-Teleman A, Bayley PM, Drakenberg T, Forsén S. 1985. Kinetics of calcium dissociation from calmodulin and its tryptic fragments. A stopped-flow fluorescence study using quin 2 reveals a two-domain structure. *Eur J Biochem* 151:543–550.
- Martin SR, Linse S, Bayley PM, Forsén S. 1986. Kinetics of cadmium and terbium dissociation from calmodulin and its tryptic fragments. *Eur J Biochem* 161:595–601.
- Nakayama S, Moncrief ND, Kretsinger RH. 1992. Evolution of EF-hand calcium-modulated proteins. *J Mol Evol* 34:416–448.
- Permyakov EA, Medvedkin VN, Mitin YV, Kretsinger RH. 1991. Noncovalent complex between domain AB and domains CD*EF of parvalbumin. *Biochim Biophys Acta* 1076:67–70.
- Potts BCM, Smith J, Akke M, Macke TJ, Okazaki K, Hidaka H, Case DA, Chazin WJ. 1995. The structure of calyculin reveals a novel homodimeric fold for S100 Ca^{2+} -binding proteins. *Nature Struct Biol* 2:790–796.
- Satishyr KA, Rao ST, Pysiaka D, Drendel W, Greaser M, Sundralingam M. 1988. Refined structure of chicken skeletal muscle troponin C in the two-calcium state at 2-Å resolution. *J Biol Chem* 263:1628–1674.
- Shaw GS, Findlay WA, Semchuk PD, Hodges R, Sykes BD. 1992a. Specific formation of a heterodimeric two-site calcium-binding domain from synthetic peptides. *J Am Chem Soc* 114:6258–6259.
- Shaw GS, Hodges RS, Sykes BD. 1992b. Determination of the solution structure of a synthetic two-site calcium binding homodimeric protein domain by NMR spectroscopy. *Biochemistry* 31:9572–9580.
- Svensson LA, Thulin E, Forsén S. 1992. Proline cis-trans isomers in calbindin D_{9k} observed by X-ray crystallography. *J Mol Biol* 223:601–606.
- Szebenyi DME, Moffat K. 1986. The refined structure of vitamin D-dependent calcium binding protein from bovine intestine. *J Biol Chem* 261:8761–8777.
- Vijay-Kumar S, Cook WJ. 1992. Structure of a sarcoplasmic calcium-binding protein from *Nereis diversicolor*, refined at 2.0 Å resolution. *J Mol Biol* 224:413–426.
- Zhang M, Tanaka T, Ikura M. 1995. Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nature Struct Biol* 2:758–767.