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Molecular mechanism of the calcium-induced conformational change in the spectrin EF-hands

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Calcium is a universally employed cytosolic messenger in eukaryotic cells. Most of the proteins that bind signalling calcium are members of the calmodulin superfamily and share two or more helix–loop–helix motifs known as EF-hands. A model, based on structure comparison of different domains and supported by preliminary NMR data, has suggested that EF-hands involved in signal transduction undergo a major conformational change upon calcium binding from a 'closed' to an 'open' state allowing protein–protein interaction. We have determined the solution structures of the EF-hand pair from α-spectrin in the absence and in the presence of calcium. The structures are in the closed and open conformation respectively, providing a definite experimental proof for the closed-to-open model. Our results allow formulation of the rules which govern the movement induced by calcium. These rules may be generalized to other EF-hands since the key residues involved are conserved within the calmodulin family.

Keywords: calcium binding/cytoskeleton/EF-hands /NMR/ tertiary structure

Introduction

Calcium is a second messenger in eukaryotic cells. At the molecular level, this implies that calcium is expected to generate conformational changes in some, if not all, of its target proteins. One of the most well characterized motifs found in a wide variety of eukaryotic calcium-binding proteins is known as the EF-hand. EF-hands have helix–loop–helix structures classified in two main sub-families (Kawasaki and Kretsinger, 1994). In canonical EF-hands, a loop 12 residues long complexes calcium by four carboxylate or carboxamide groups and a single backbone carbonyl oxygen. The loop is extended to 14 residues in pseudo EF-hands and complexes the ion through a side chain carboxylate group and four backbone oxygens. In both cases, the liganding oxygens build a pentagonal bipyramid around the calcium ion. In all the structures determined so far, EF-hands occur in side-to-side pairs which form either one (parvalbumin and calbindin) or two (calmodulin, troponin C, recoverin, myosin essential and regulatory light chains) globular units (Kawasaki and Kretsinger, 1994). Each unit can bind up to two calcium ions, one in each helix–loop–helix motif.

EF-hand structures are found in two conformations, often referred to as 'closed' and 'open' (Hertzberg *et al.*, 1986). They differ mainly in the relative angles between helices and consequently in a different pattern of helix packing. Generally, the closed and open conformations correspond to calcium-free and calcium-bound states respectively. Based on this observation, a unifying model for the calcium-induced conformational change was proposed by Herzberg *et al.* (1986). In the absence of calcium, the four helices would pack in pairs forming a very compact structure (closed state). Upon binding of calcium, the two helices of each EF-hand motif would be pushed apart inducing the exposure of hydrophobic residues otherwise involved in helix–helix packing in the calcium-free form. These residues would then become available for interaction with the target protein.

Increasing experimental support for this closed-to-open transition has been accumulating over the last years (Finn *et al.*, 1993; Gagné *et al.*, 1994). The structures of different calmodulin complexes with amphipathic target peptides (Ikura *et al.*, 1992; Meador *et al.*, 1992) have confirmed the role in binding of the exposed residues. However, no structure of the same EF-hand pair has been published so far which adopts a closed state in the absence of calcium and an open state in the calcium-bound state. The only structure currently known in the absence and in the presence of calcium is calbindin, composed of a canonical and a pseudo EF-hand (Skelton *et al.*, 1990, 1994). However, no conformational transition is observed in calbindin and the structure remains in a closed conformation in both states.

We have begun the structural characterization of the C-terminus of chicken α-spectrin (Travé *et al.*, 1995). We have demonstrated the existence of an independently folded domain which spans 159 residues at the very end of the α-spectrin chain. Our data led us to predict for this domain a calmodulin-like structure, with two globular units. Only the N-terminal domain contains two canonical EF-hands sequences which were found to bind specifically two calcium ions, although with low, non-physiological affinity *in vitro*. We present here the structure determination of this active EF-hand domain in the absence and in the presence of calcium. The protein is found to undergo the conformational change predicted by Herzberg *et al.* (1986). The comparison of the structures in the two forms enables us to deduce the structural basis of the functional mechanism induced by calcium binding to the EF-hands.

Results

The sequence of the protein construct studied in this work is shown in Figure 1. It contains two EF-hands and spans

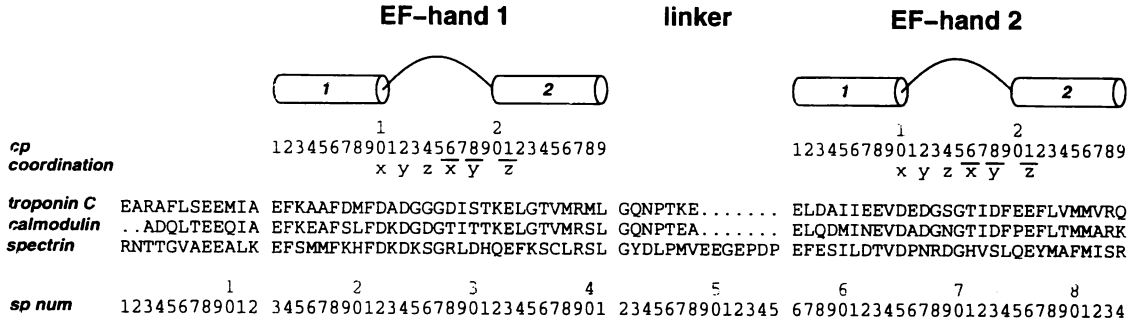

Fig. 1. Primary sequence of the α-spectrin EF-hand domain. The secondary structure of the two helix–loop–helix EF-hand motifs is indicated, as well as the letter code for calcium ligands. The two EF-hands are aligned with the N-terminal EF-hands of troponin C and calmodulin. Both canonical numbering proposed by Kawasaki and Kretsinger (1994) (cp, top line) and the construct numbering (sp num, bottom line) are indicated.

from Arg2320 to Arg2403 of the chicken α-spectrin sequence (Wasenius *et al.*, 1989), with an additional Met at its N-terminus (not included in the construct numbering). Canonical positions within each EF-hand (indicated hereon as cp) according to Kawasaki and Kretsinger (1994) are indicated to facilitate comparison of corresponding positions in the two EF-hands. For comparison, the sequences of the EF-hand regions of calmodulin and troponin C are also shown. In spectrin, the linker region connecting the two EF-hands has fourteen residues instead of seven in other prototypical EF-hand proteins. The design, cloning and purification of the domain have already been described elsewhere (Travé *et al.*, 1995). The protein is extremely soluble, since it can be concentrated up to 10 mM without any detectable precipitation or aggregation (not shown). The domain was shown to bind two calcium ions with low affinity but high specificity (Travé *et al.*, 1995).

Assignment of the NMR spectra

NMR spectra of excellent quality were obtained for the protein in the absence and in the presence of calcium which allowed complete sequential assignment of the two forms. The spectrum of the calcium-free form was assigned using 2D TOCSY and NOESY supported by 3D ^{1}H-^{15}N TOCSY- and NOESY-HSQC experiments. According to standard procedures (Wüthrich, 1986), comparison of NOESY connectivities between backbone and side chain protons (Figure 2A) with peaks observed in TOCSY and COSY experiments allowed identification of all the spin systems, with the exclusion of the first five residues. Problems of overlap mainly arising from the intrinsic poorer dispersion of the all-helical secondary structure were solved using the 3D hetero-nuclear experiments, taking advantage of the higher dispersion of the ^{15}N frequencies (Figure 2B). The assignment was easily extended to the calcium-bound form, for which only 2D experiments were used, exploiting the similarities to the spectra of the calcium-free form.

Summaries of the experimental short- and medium-range NOE distances for the two forms are shown in Figure 3. The NOE connectivity pattern is typical of the EF-hand fold. HN–HN(i, i + 1), HN–HN(i, i + 2), Hα–HN(i, i + 3), Hα–HN(i, i + 4) and Hα–Hβ(i, i + 3) connectivities allow localization of four helices encompassing residues 9–21, 31–42, 56–65 and 74–83 respectively. Long range β-sheet connectivities are found between residues 27–29 and 70–72, revealing the charac-

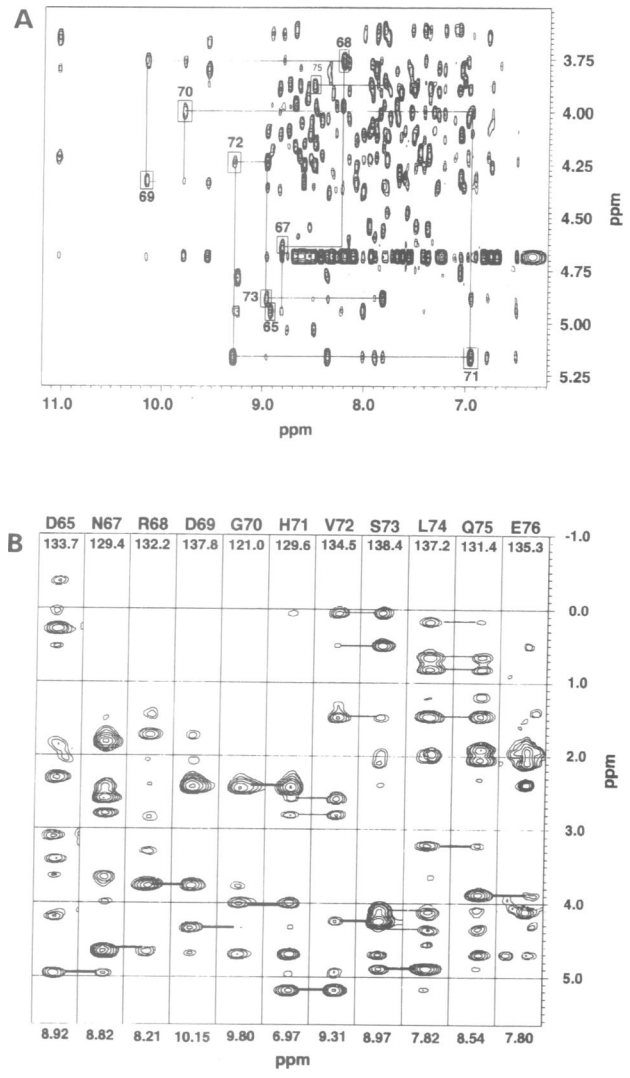

Fig. 2. Representative spectral regions of experiments recorded at 300 K and 600 MHz on the calcium-free form of the spectrin domain. The quality of the experiments carried out on the calcium-bound form is comparable. (**A**) Fingerprint region of a 2D NOESY experiment (80 ms mixing time). Intra- and inter-residue connectivities are indicated for the sequence from Asp65 (cp 10) to Glu76 (cp 21). Connectivities involving the amide of residue 66 are missing since this residue is a proline. (**B**) 1D strips from a 3D ^{1}H-^{15}N NOESY-HSQC experiment (100 ms mixing time) for the same stretch as in (A).

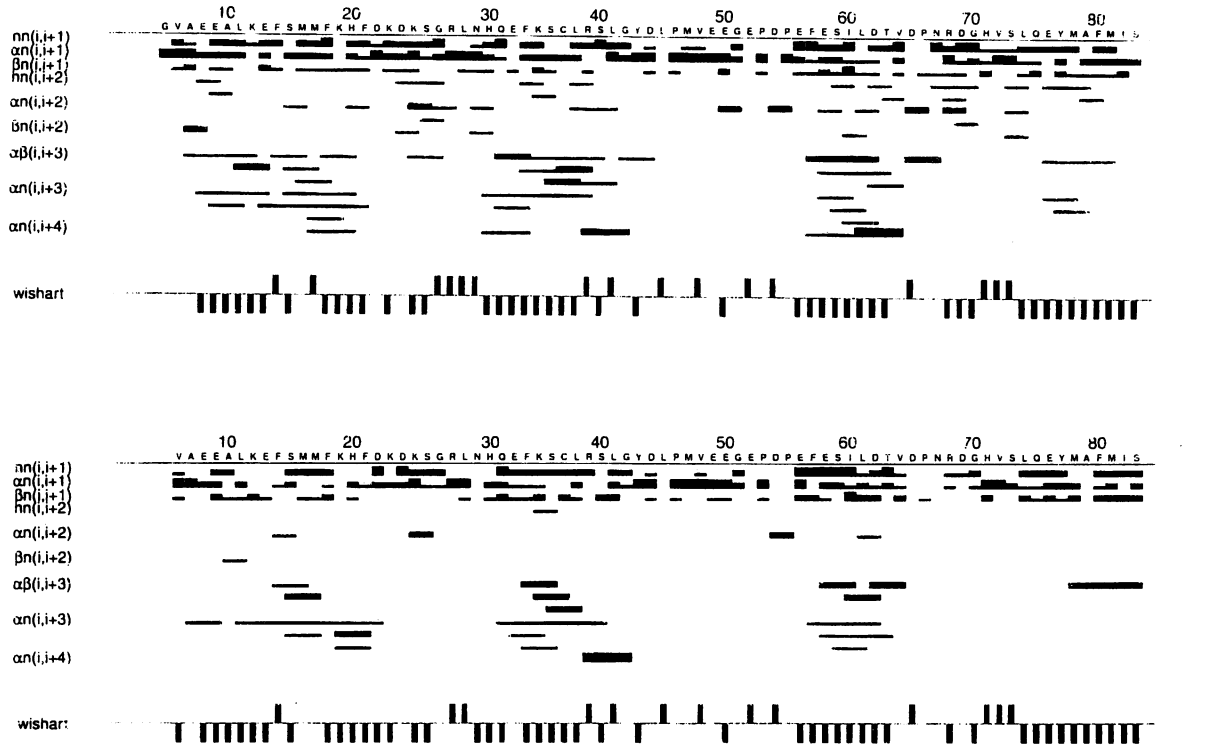

Fig. 3. Surveys of short and medium range NOEs for the calcium-free (top) and the calcium-bound form (bottom). The first five residues are not reported since they are unstructured and do not show NOESY connectivities. Chemical shift index for the α-protons as defined by Wishart *et al.* (1992) are reported in the last row. Negative values indicate helical regions, positive values extended regions.

teristic anti-parallel β-sheet responsible for pairwise loop–loop interactions (Strynadka *et al.*, 1989).

Chemical shift analysis

Backbone chemical shifts have long been known to be markers of protein secondary structure (Pastore and Saudek, 1990; Wishart *et al.*, 1991). It is therefore reasonable to expect that the very similar structure of the EF-hands will be reflected by a reproducible chemical shift pattern for these proteins. Furthermore, a reproducible chemical shift variation induced by similar conformational changes of the loop upon calcium binding will be expected among members of the EF-hand family. Figure 4 shows plots of the α-proton chemical shift differences between the calcium-free and the calcium-bound state versus the sequence as determined for each of the two EF-hands of the spectrin domain and for three other prototypical EF-hands (the average over the chemical shift values published for the three motifs is reported; Koerdel *et al.*, 1989; Gagné *et al.*, 1994). The changes occurring in EF-hand 1 are extremely similar to the averaged values, suggesting that this loop undergoes a conformational change representative of what generally happens in the family. EF-hand 2 shows a less typical pattern. This could be related to the presence of a proline (Pro66, cp 11) of this EF-hand. The proline is flanked by two liganding positions of the calcium ion and may consequently influence the ion-induced movement.

Structure determination

Structure calculations were performed using a combination of distance geometry and molecular dynamics. 693 and 567 distance restraints were obtained from the 2D and 3D

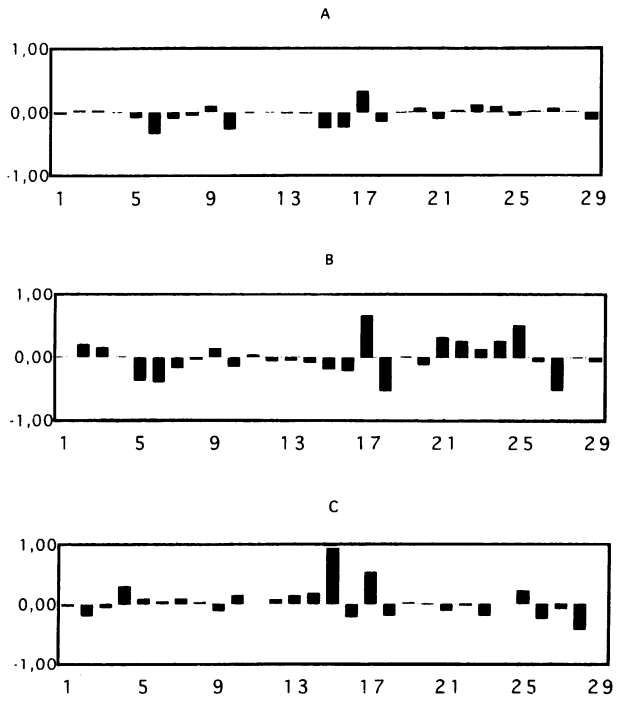

Fig. 4. Difference of the Cα proton chemical shifts as obtained in the calcium-free and calcium-bound forms. The numbering is given according to the canonical positions along each EF-hand. (**A**) Average of the calcium-induced chemical shift changes published for EF-hands 1 and 2 of the N-terminus of troponin C (Gagné *et al.*, 1994) and for EF-hand 2 of calbindin (Koerdel *et al.*, 1989). (**B**) Calcium-induced chemical shift changes in spectrin EF-hand 1. (**C**) Calcium-induced chemical shift changes in spectrin EF-hand 2.

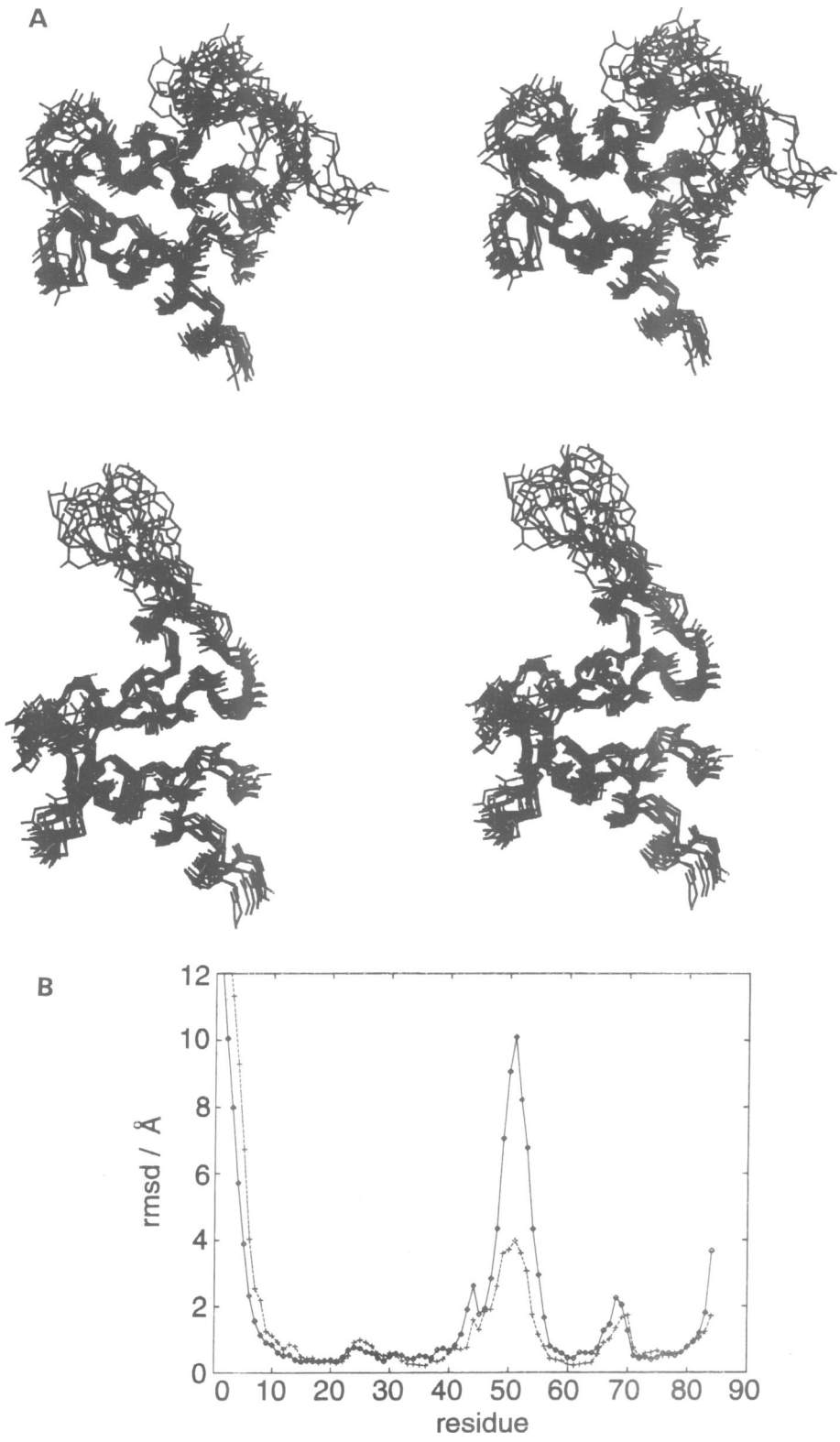

Fig. 5. (**A**) Stereo representations of the superposition of the 10 best structures obtained for the spectrin domain in the absence (top) and in the presence (bottom) of calcium. Backbone N, C, O and Cα atoms of residues 8–84 are shown. (**B**) R.m.s. deviations from the average structure as reported versus the sequence. Diamonds and crosses are used for the calcium-free and the calcium-bound form respectively.

NOESY spectra for the calcium-free and calcium-bound forms respectively. Forty-five hydrogen bond distances deducible from the observed α-helical secondary structures were also added at advanced stages of the calculations (see Materials and methods). Ensembles of 10 final structures calculated for both forms are shown in Figure 5A and B. Average root-mean-square (r.m.s.) deviations of 0.55 and 0.46 Å were obtained for the backbone atoms in the helical regions for the calcium-free and calcium-bound forms respectively (Figure 5B). Calcium binding has no

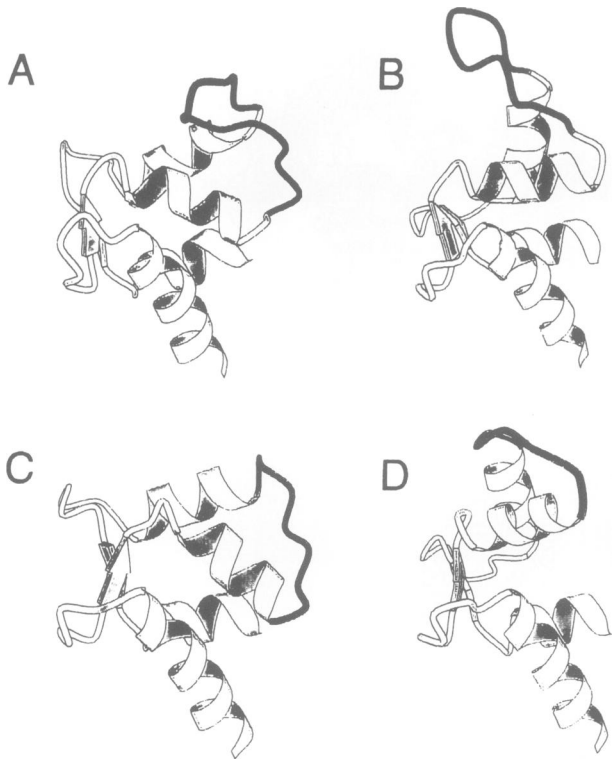

Fig. 6. MOLSCRIPT representation of the structures of the calcium-free (**A**) and calcium-bound form (**B**) of the spectrin EF-hand domain. The structures with the best r.m.s. deviation from the average were chosen and displayed in the same orientation as in Figure 5A. For comparison, the structures of the N-terminal domain of troponin C (**C**) and of calmodulin are also reported (**D**). The loops connecting the EF-hands are indicated in black.

major influence on the r.m.s. deviation of the calcium-binding loops in each EF-hand. However, the two calcium-binding loops are less well defined. Particularly EF-hand 2 is affected: the r.m.s. deviation in the first loop (residues 22–30) is <0.7 Å, while it is between 1 and 2 Å in the second loop (residues 67–71). The linker connecting the two EF-hands (residues 45–55) has the highest values of r.m.s. deviation especially in the calcium-free form (Figure 5B). Together with other NMR parameters (the secondary chemical shifts are close to random coil values and residues in this region form almost no NOE contacts with the surrounding), this suggests that this region is in a disordered conformation.

Side chains within the protein core are very well defined. This is related to the large number of restraints (102 for the calcium-free form, 87 for the calcium-bound form) involving the six aromatic rings of the core: Phe14, Phe18, Phe21, Phe34, Phe57 and Tyr77 (which correspond to cp 2, 6, 9, 22 of EF-hand 1 and 2 and 22 of EF-hand 2).

Structure comparison with other EF-hands

MOLSCRIPT representations (Kraulis, 1991) for the calcium-free and calcium-bound forms of the spectrin EF-hand domain are shown in Figure 6A and B. Comparison of the two structures shows that binding of calcium induces dramatic conformational changes. In the absence of calcium, the conformation is closed and similar to the N-terminal calcium-free domain of troponin C (Sundaralingam *et al.*, 1985; Figure 6C). In the presence of calcium, the conformation is open and reminiscent of the calcium-bound domains of calmodulin (Figure 6D) and troponin C (Babu *et al.*, 1985; Sundaralingam *et al.*, 1985).

In comparison with both calmodulin and troponin C, helix 3 is slightly shorter (two residues) most likely as a consequence of two prolines (Pro53 and 55, see Figure 1). As a consequence, the linker region which connects the two EF-hands extends from Gly42 to Pro55. Along the linker, a stretch of four hydrophobic residues, LPMV, is fully exposed to the solvent. The unusual exposure of this hydrophobic stretch within a flexible region makes the linker an interesting candidate for protein–protein interaction.

Redistribution of inter-helix contacts

According to the model of Herzberg *et al.* (1986), the main differences between closed and open forms are expected both at the level of a variation of the helix packing pattern and of a much higher compactness of the closed form as compared with the open form. Major changes are observed in the exposure of hydrophobic residues of the spectrin domain in the two forms (Figure 7A and B), with a net accessibility gain of 350 $Å^2$ upon calcium binding (as calculated only for the two EF-hand regions, i.e. 8–41 and 55–83).

Calcium induces an important redistribution of the helix contacts among the four helices. Figure 7C shows the changes occurring both within each individual EF-hand and between each other. Vertical inspection of the three plots on the left (for the calcium-free form) shows that, in the absence of calcium, a much tighter internal packing is observable in EF-hand 1 (helices 1 and 2) as compared with EF-hand 2 (helices 3 and 4). Several hydrophobic contacts provide a tight core in EF-hand 1 while EF-hand 2 is practically devoid of internal contacts. The hydrophobic residues in the calcium-free EF-hand 2 are instead much more involved in side-to-side interactions with EF-hand 1 (see bottom left plot). EF-hand 1 is therefore in a closed conformation as a consequence of its own tight internal contacts, whereas EF-hand 2 is closed because of its tight side-clamping to EF-hand 1.

These differences tend to disappear upon calcium binding. Horizontal comparison of the plots in Figure 7C illustrates the effects of the calcium-induced reorganization on internal contacts, especially in EF-hand 2. Several hydrophobic contacts between the N-terminus of helix 1 and the C-terminus of helix 2 disappear in EF-hand 1. In EF-hand 2, a whole set of new contacts is formed. As a result, the two calcium-bound EF-hands display a similar pattern of contacts mainly involving the immediate boundaries of the helices with the calcium-binding loops. These observations confirm the model first proposed by Hertzberg *et al.* (1986).

Key residues

Hydrophobic contacts are subject to important redistributions in both EF-hands upon calcium binding. However, four hydrophobic residues in EF-hand 1 constitute a core which remains unaffected by calcium binding: Phe21 (cp 9) which tightly packs against Cys37 (cp 25) and Phe18 (cp 6) which packs against Leu29 (cp 17). These four residues connect helices 1 and 2 to the short β-

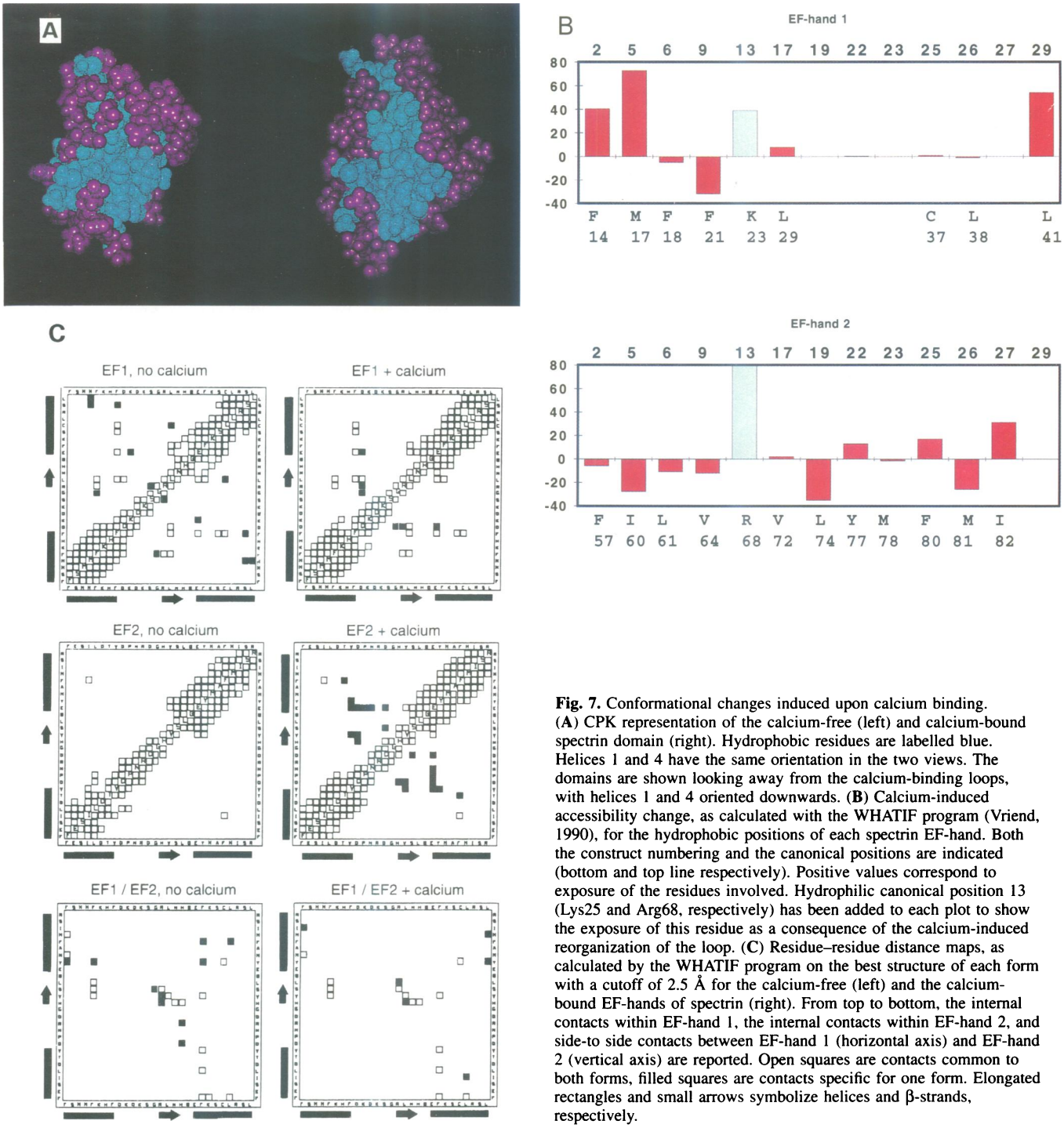

Fig. 7. Conformational changes induced upon calcium binding. (**A**) CPK representation of the calcium-free (left) and calcium-bound spectrin domain (right). Hydrophobic residues are labelled blue. Helices 1 and 4 have the same orientation in the two views. The domains are shown looking away from the calcium-binding loops, with helices 1 and 4 oriented downwards. (**B**) Calcium-induced accessibility change, as calculated with the WHATIF program (Vriend, 1990), for the hydrophobic positions of each spectrin EF-hand. Both the construct numbering and the canonical positions are indicated (bottom and top line respectively). Positive values correspond to exposure of the residues involved. Hydrophilic canonical position 13 (Lys25 and Arg68, respectively) has been added to each plot to show the exposure of this residue as a consequence of the calcium-induced reorganization of the loop. (**C**) Residue–residue distance maps, as calculated by the WHATIF program on the best structure of each form with a cutoff of 2.5 Å for the calcium-free (left) and the calcium-bound EF-hands of spectrin (right). From top to bottom, the internal contacts within EF-hand 1, the internal contacts within EF-hand 2, and side-to side contacts between EF-hand 1 (horizontal axis) and EF-hand 2 (vertical axis) are reported. Open squares are contacts common to both forms, filled squares are contacts specific for one form. Elongated rectangles and small arrows symbolize helices and β-strands, respectively.

strand through Leu29 (cp 17). A third pair of interacting hydrophobic residues, Met17 (cp 5) and Leu41 (cp 29), is present in calcium-free spectrin domain but breaks in the calcium-bound form. The calcium-induced change of accessibility upon calcium binding for the key residues of the two EF-hands is detailed in Figure 7B. The plot traces the dramatic exposure of Met17 (cp 5) and Leu41 (cp 29) to solvent, whilst Phe18 (cp 6), Phe21 (cp 9), Leu29 (cp 17) and Cys37 (cp 25) are either not affected or become slightly more buried in the calcium-bound form. Such a constant core is absent in EF-hand 2, where the two helices are too distant to pack against each other in the calcium-free form.

Analysis of the two structures also shows another contact which seems to play an important role in determining the structure of EF-hand 1. In calcium-free protein, Lys23 (cp 11) is close enough to the calcium ligand Glu33 (cp 21) to establish a salt bridge with it. This result is supported by several unambiguous NOE distances involving residues Asp22, Lys23, Leu29 and Glu33 (cp 10, 11, 17 and 21). In all calculated structures, the minimal distance between two atoms of these residues is <3.5 Å. In calcium-bound spectrin the two aspartic side chains which surround Lys23 (cp 11) reorient in the direction of the ion. The salt bridge is broken and the loop has a more extended conformation.

Discussion

Function of the EF-hand domain in spectrin

We have presented here the structures of the spectrin EF-hand domain in the absence and in the presence of calcium. The two structures have all the features expected for a closed and an open form respectively. The precise function of the EF-hand domain in spectrin is not known. α-Actinin, a protein highly homologous to spectrin, is assembled in an antiparallel homodimer of two identical subunits. In some forms of α-actinin, the C-terminal EF-hand domain has been shown to modulate the activity of the N-terminal actin-binding domain, present on the opposite chain in response to calcium (Taylor and Taylor, 1993; Witke *et al.*, 1993). In spectrin, two elongated α and β subunits are associated side-to-side to form an anti-parallel heterodimer (Bennett, 1990; Luna, 1991). Mutagenesis data published by Viel and Branton (1994) indicate that the α-spectrin C-terminus is one of the multiple sites which mediate self-association between the α and β chains. A detailed analysis of the results published by Viel and Branton shows however, that all the mutants which contain the linker region between EF-hands preserve self-association, irrespective of the presence of the neighbouring EF-hands. This, together with our results according to which the loop is mainly unstructured in solution with an exposed patch of hydrophobic residues, leads us to suggest that it is the linker region which is responsible for the inter-chain contacts involving this part of the molecule. Whether, as observed in α-actinin, this interaction leads to calcium modulation of actin-binding properties remains to be assessed by biochemical analysis.

However, our results go beyond the specific interest of the structure of the spectrin domain since they provide a model of the mechanism of calcium-induced conformational changes common to the whole family of calmodulin-like proteins.

The mechanism of calcium binding

Detailed comparison of the two forms allows us to describe a general mechanism of the closed-to-open transition. In the absence of calcium, three pairs of hydrophobic interactions provide a tight internal core in EF-hand 1 (Figure 8A): Phe21–Cys37 (cp 9/25), Phe18–Leu29 (cp 6/17) and Met17–Leu41 (cp 5/29). Lys23 (cp 11) is close enough to the calcium-ligand Glu33 (cp 21) to establish a salt bridge with it. This salt bridge gives a 'closed' shape to the loop. In EF-hand 2, the two helices are further from each other, and internal interactions are looser than in EF-hand 1 (Figure 8B). The majority of hydrophobic residues are involved in side-to-side interactions with EF-hand 1 (Figure 7C).

Upon calcium binding, the major movement occurs at EF-hand 1. The structure of the calcium-binding loop is completely reorganized. The calcium chelating residues, i.e. Asp22, Asp24, Ser26 and Glu33 (cp 10, 12, 14 and 21), are forced to point towards each other. The salt bridge breaks, and the loop stretches (downwards in Figure 8A). Its extension induces a kink of the helix at Phe18 (cp 6) which lifts up the aromatic side chain of Phe21 (cp 9). This rotation propagates to helix 2 through Cys37 (cp 25) which packs against the ring of Phe21 (cp 9). The whole helix 2 rotates as a rigid body around a perpendicular axis (indicated with a curved arrow in Figure 8A). The movement breaks the hydrophobic pair Met17–Leu41 (cp 5/29), but does not affect the other two hydrophobic interactions. As the loop extends downwards, Glu33 (cp 21), the fourth calcium-chelating residue, is also forced to point in the same direction. EF-hand 2 is also reorganized upon calcium binding (Figure 8B). However, since the strong hydrophobic core and the two aromatic residues essential for the movement of EF-hand 1 are absent, the movement of EF-hand 2 propagates from EF-hand 1 through side-to-side packing.

The analysis of the spectrin EF-hands therefore provides a general scheme for the conformational change: EF-hand 1 is the motor and EF-hand 2 acts as a transistor which cannot generate the movement but may be able to modulate the affinity of the whole domain through cooperative effects.

Validity of the mechanism for other EF-hands

The key aromatic residues specific to EF-hand 1 are conserved in many members of the calmodulin family, so is the replacement of these aromatic residues by short aliphatic side chains in the EF-hand 2. Therefore, the rules of conformational change may be useful to predict and understand the behaviour of other members of the EF-hand family.

In the *Dictyostelium* α-actinin, mutagenesis studies provide strong support to our 'motor–transistor' analysis of the role of the two EF-hands (Witke *et al.*, 1993). These results indicate that EF-hand 1 binds calcium poorly but is essential for biological activity, i.e. cross-linking of F-actin. EF-hand 2 has a higher affinity, but is not required for activity. In calbindin, the motor EF-hand 1 is replaced by a pseudo EF-hand where all chelating positions are already correctly placed in the absence of calcium (Skelton *et al.*, 1990, 1994). This would fully explain why the conformation of calbindin is hardly affected by calcium.

The calcium-free EF-hand 1 of troponin C (Sundaralingam *et al.*, 1985) provides an interesting variation from what is observed in the spectrin EF-hand 1, with features intermediate between the conformations of the two forms of the spectrin domain. The first two pairs of residues which constitute the hydrophobic core are conserved. However, lysine at cp 11 is absent. The salt bridge cannot be formed, resulting in a loop shape more comparable with that of the calcium-bound form of spectrin EF-hand 1 (Figure 8). As in the calcium-bound form of the spectrin EF-hand domain, the extended conformation of the loop forces the aromatic side chain at cp 9 to be lifted up. However, instead of being rotated upwards, helix 2 of troponin C maintains the hydrophobic pair cp 5/29. This is allowed by a kink at cp 21, clearly observable in the crystal structure (Sundaralingam *et al.*, 1985). In both forms of the spectrin domain, helix 2 is straight. The difference must be related to the fact that cp 21 (Glu33) is free in troponin C but stretches towards the ion in the calcium-bound spectrin EF-hand 1. Confirming this analysis, a recent NMR study (Gagné *et al.*, 1994) shows that the main change induced by calcium in the secondary structure of the N-terminal domain of troponin C is a straightening of the helix at cp 21. Interestingly, a lysine is found at cp 11 of EF-hand 1 in several domains of the family which bind calcium with a high affinity. These

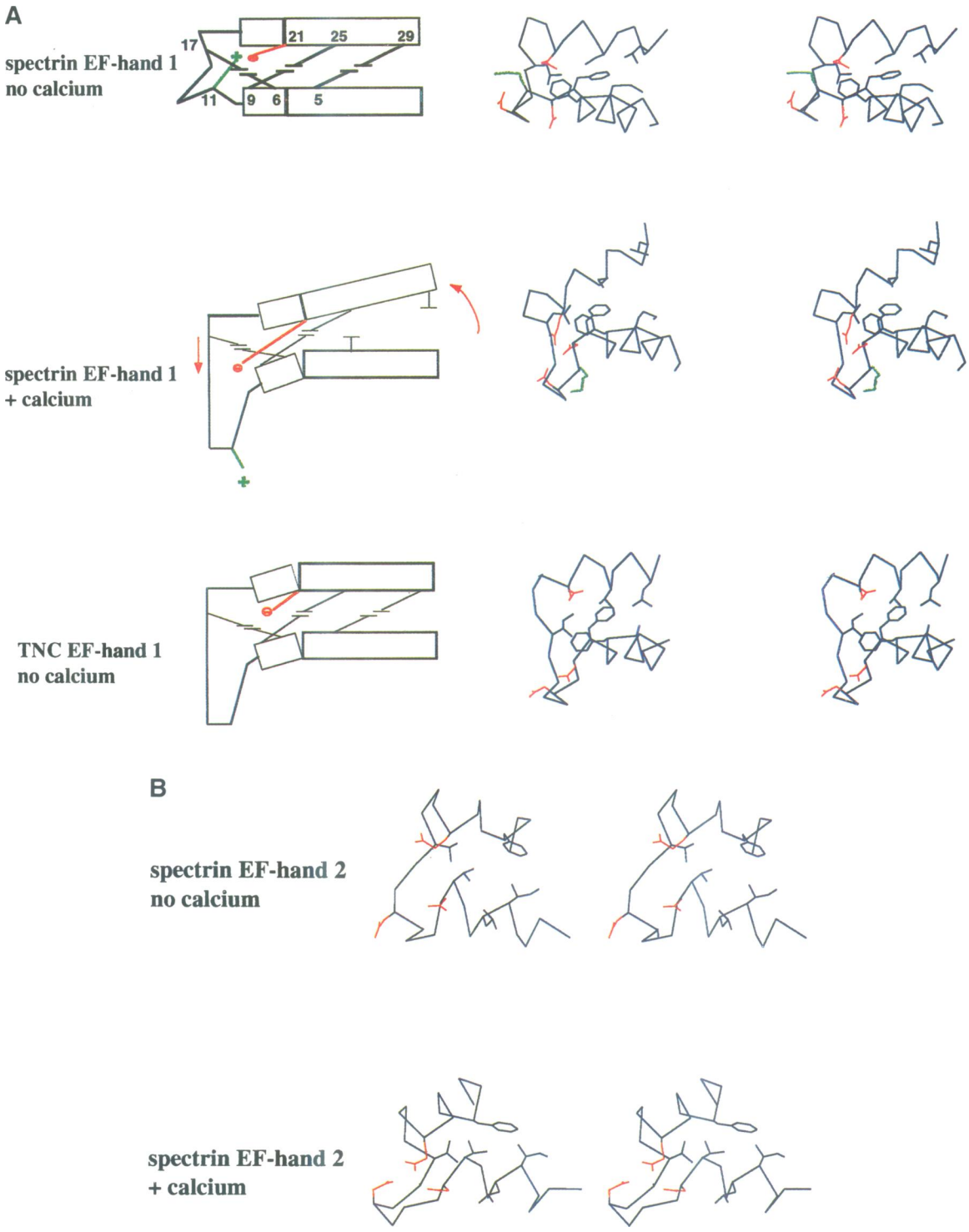

Fig. 8. Description of the detailed mechanism of the closed-to-open transition. (**A**) Stereo views of the EF-hand 1 of the calcium-free spectrin domain (top), the calcium-bound form of spectrin (middle) and the calcium-free form of troponin C (bottom). In the stereoview, acidic, basic and hydrophobic side chains are coloured in red, green and blue, respectively. The Cα trace is coloured in blue. Schematic sketches on the left summarize features important for the movement. In the scheme, the three pairs of hydrophobic residues essential for the movement of EF-hand 1 [Phe21–Cys37 (cp 9/25), Phe18–Leu29 (cp 6/17) and Met17–Leu41 (cp 5/29)] are indicated in the scheme as straight lines interrupted by horizontal bars and labelled by their canonical positions. Plus and minus signs indicate the side chains of Lys23 (cp 11) and Glu33 (cp 21). A straight red arrow indicates the breakage of the salt bridge and the movement of the loop downwards. The extension of the loop induces a kink in helix 1 around canonical position 6 (indicated by an interruption of the left part of the rectangle representing helix 1). A red curved arrow indicates the change of opening rotation upwards of helix 2 induced by the change of orientation of aromatic ring of Phe21 (cp 9). For comparison, the structure of the calcium-free form of EF-hand 1 from troponin C N-terminal domain is shown (Sundaralingam *et al.*, 1985). An additional kink is present in helix 2 at cp 21, and hydrophobic pair cp 5/29 is conserved. (**B**) Stereoviews of the EF-hand 2 of calcium-free spectrin domain (top) and calcium-bound spectrin domain (bottom). Both orientation and colour code are the same as in (A) for EF-hand 1. To stress differences with EF-hand 1, the side chains of canonical positions 5, 6, 9, 10, 12, 17, 21 and 25 are shown. They correspond to residues Ile60, Leu61, Val64, Pro66, Val72, Glu76 and Phe80 respectively.

include both globular units of calmodulin and the C-terminal domain of troponin C. The salt bridge observed in the spectrin EF-hand, and the subsequent loop shape, may be expected to be present in the calcium-free form of these domains. If this is true, the conformational change of these domains should be closer to the one described here for the spectrin EF-hands than to that proposed for the N-terminal domain of troponin C.

The structures in the absence and in the presence of calcium of other members of the calmodulin family are expected. Extensive comparison between the different structural solutions will be very interesting and will undoubtedly increase our understanding of molecular details of calcium activation. The basic rules presented here already provide a guideline to correlate the presence in an EF-hand sequence of the key residues with expected calcium-binding conformational changes. This may inspire the design of mutations to affect independently either calcium affinity or ion-induced activity.

Materials and methods

The design, cloning and purification of the construct have been described elsewhere (Travé *et al.*, 1995). ^{15}N-enriched samples were obtained by growing cells using a minimal medium with $^{15}NH_4Cl$ as the sole nitrogen source. NMR spectra were recorded on 2–4 mM samples in 90% H_2O/10% D_2O and containing 100 mM KCl at pH 7, in the absence or in the presence of calcium (10 mM).

All spectra were recorded at 600 MHz ^1H frequency on an AMX-BRUKER spectrometer in the temperature range 290–310 K. The spectra were acquired in phase sensitive mode (TPPI) either with pre-irradiation of the water resonance or with selective excitation and pulsed-field gradients (WATERGATE pulse sequence, Piotto *et al.*, 1992; Sklènar *et al.*, 1993). 2D spectra were recorded using 2048 data points in the acquisition domain and 512 data points in t_1. TOCSY spectra were measured using the TOWNY composite pulse cycle (Kadkhodaei *et al.*, 1993). Mixing times used were in the 50–100 ms range for the TOWNY and 80–200 ms for the NOESY experiments. Prior to Fourier transformation, the data were zero-filled to 2048 points in the t_1 dimension and weighted with a Gaussian window in t_2 and a sine window in t_1. A baseline correction was performed in both dimensions using a polynomial. Data were processed on a Bruker X-32 station using the UXNMR program. ^{15}N-^1H 2D HSQC, 3D NOESY-HSQC (100 ms mixing time) and 3D TOWNY-HSQC (50 ms mixing time) spectra were recorded as published by Sklènar *et al.* (1993) at a temperature of 300 K. Using TPPI in the two indirect dimensions of the 3D experiments a total of 180 real points were recorded for ^1H and 84 real points for ^{15}N with 1024 points in the acquisition dimension. 3D spectra were processed using NMR3D, kindly provided by Drs C.Cieslar (Munich) and H.Oschkinat (Heidelberg). The two indirect dimensions were extended from 180 to 256 for ^1H and 84 to 128 real points for ^{15}N, respectively, by linear prediction and zero filled to 512 and 256 points prior to multiplication with a Gaussian function and subsequent Fourier transformation. Processed spectra were analysed and plotted using AURELIA (Bruker AG, Karlsruhe) on a SGI Indigo workstation. List of the assignment is available as additional material.

Distances were obtained from the 2D NOESY spectra at 80 ms at 300 K and calibrated by the AURELIA program (Bruker, Karlsruhe) assuming a distance of 2.7 Å for the HN34–HN35 crosspeak. The consistency of this choice with expected intra-residue distances was checked. Additional restraints were obtained from the 3D NOESY hetero-nuclear experiment calibrated in the same way. A total of 693 and 567 restraints were used for the calcium-free and calcium-bound forms respectively. Of these, respectively, 295 and 241 were sequential, 205 and 215 short-range, 36 and 31 medium-range, and 92 and 80 long-range.

Structure calculations were performed by a combination of distance geometry and molecular dynamics. First, three cycles of the REDAC strategy were performed using the DIANA program (Güntert and Wüthrich, 1991; Güntert *et al.*, 1991). 45 hydrogen bond distances for α-helices detected in the first runs were added in the last REDAC run. No hydrogen bonds were explicitely imposed for the β-sheet. Additional distances defining the ideal calcium-complexing pentagonal bipyramide were added for the liganding residues of the calcium-bound form. Parallel calculations were done without the addition of these distances to check that they did not influence the global fold, but simply the orientation of the chelating residues. The best 100 structures obtained from DIANA were subsequently refined with the XPLOR program (Bruenger, 1992) adding explicit hydrogen bond terms to a standard simulated annealing protocol. After heating to 3000 K for 20 ps, the system was cooled down in 30 ps to 300 K. The temperature was kept constant for 5 ps after which the structure was energy-minimized for 100 steps. For each form, 10 structures showing no violation larger than 0.5 Å and a maximal total energy of 300 kcal/mol were kept. The structures were superimposed using solely the backbone atoms in the helical regions (residues 10–21, 33–41, 57–63 and 76–83), and an average structure was calculated (Diamond, 1992). The coordinates will be deposited with the Brookhaven Protein Data Bank.

Graphics support was obtained by the WHATIF program (Vriend, 1990).

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Note added in proof

After submitting this paper, we became aware of the existence of other papers currently in press describing the structure of the EF-hand domains in the presence and in the absence of calcium.