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Structure of Ca^{2+}-loaded human grancalcin

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Grancalcin is a cytosolic Ca^{2+}-binding protein originally identified in human neutrophils. It belongs to a new class of EF-hand proteins, called PEF proteins, which contain five EF-hand motifs. At the N-terminus of grancalcin there is a ~50 residue-long segment rich in glycines and prolines. The fifth EF-hand, unpaired within the monomer, provides a means for dimerization through pairing with its counterpart in a second molecule. The structure of full-length grancalcin in the apo form and with one EF3 within the dimer occupied by a Ca^{2+} ion have been determined. Although the N-terminal segment was present in the molecule, this part was disordered in the crystals. Here, the structure of a truncated form of grancalcin, which is lacking 52 N-terminal residues, in the presence and absence of Ca^{2+} is presented. In the Ca^{2+}-bound form the ions are found in the EF1 and EF3 hands. Binding of Ca^{2+} to these two EF hands produces only minor conformational changes, mostly within the EF1 Ca^{2+}-binding loop. This observation supports the hypothesis, formulated on the basis of the structure of a homologous protein ALG-2 which shows significant differences in the orientation of EF4 and EF5 compared with grancalcin, that calcium is a necessary factor but not sufficient alone for inducing a significant conformational change in PEF proteins.

1. Introduction

Grancalcin is a cytosolic calcium-binding protein originally identified in human neutrophils (Teahan *et al.*, 1992; Lollike *et al.*, 1995). It has been found in cells of hematopoietic origin, including monocytes, with relatively small amounts present in lymphocytes (Boyhan *et al.*, 1992). In the presence of calcium, grancalcin binds reversibly to secretory vesicles and plasma membranes and is suspected to play a role in the regulation of vesicle/granule exocytosis (Borregaard *et al.*, 1992; Lollike *et al.*, 1995). Recently, L-plastin (L stands for leukocyte isotype) was identified as a binding partner for grancalcin (Lollike *et al.*, 2001). Plastins are EF-hand proteins with actin-binding motifs and they are known to have actin-bundling activities (Jones *et al.*, 1998). Grancalcin associates with L-plastin only in the absence of calcium, whereas this interaction is inhibited in the presence of calcium (Lollike *et al.*, 2001).

Grancalcin belongs to the penta-EF-hand (PEF) family of calcium-binding proteins. Other members of this protein family are the large and small subunit of calpain, sorcin, the apoptosis-linked protein ALG-2 and peflin (Maki *et al.*, 1997). These proteins contain five EF-hand motifs attached to an N-terminal region of variable length containing one or more short Gly/Pro-rich sequences. The molecules form dimers through pairing between the fifth EF-hands from the two

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molecules (Blanchard *et al.*, 1997; Lin *et al.*, 1997; Kretsinger, 1997). The arrangement of the pairs of EF-hands is characteristic of this family and differs from other EF-hand-containing Ca^{2+}-binding proteins (Yap *et al.*, 1999). Like grancalcin, other PEF proteins are also cytosolic proteins that reversibly associate with cell membranes at physiological Ca^{2+} concentrations (Zamparelli *et al.*, 1997). The structure of the Ca^{2+}-binding domain of calpain light chain, the prototypic PEF protein, has been determined in the presence of Ca^{2+} (Blanchard *et al.*, 1997; Lin *et al.*, 1997) and in its absence (Blanchard *et al.*, 1997). Only rather small conformational rearrangements were observed in this domain upon binding of Ca^{2+}, localized primarily in the EF1 hand. How these Ca^{2+}-induced changes contribute to the regulation of calpain activity is not yet clear, even with the recently determined structure of the entire calpain molecule in the absence of Ca^{2+} (Hosfield *et al.*, 1999; Strobl *et al.*, 2000). We have recently determined the structure of another protein belonging to the PEF family, ALG-2, in the presence of Ca^{2+} (Jia *et al.*, 2001). Intriguingly, we have found a molecule bound within a surface groove created between EF2 and EF4, which we interpreted as a Gly/Pro-rich decapeptide. Comparison of ALG-2 with calpain showed a different arrangement of EF1/EF2 relative to EF4/EF5 in these two proteins. The conformation observed in ALG-2 created the peptide-binding groove and we hypothesized that calcium sensing requires a combined effect of Ca^{2+} and the binding of a specific peptide (Jia *et al.*, 2001).

Grancalcin exists as dimers in solution. Evaluation of Ca^{2+} binding to recombinant grancalcin by flow dialysis revealed two Ca^{2+}-binding sites per monomer, with an affinity in the medium micromolar range (Lollike *et al.*, 1995, 2001). Furthermore, tryptophan fluorescence indicated that upon Ca^{2+} binding some conformational rearrangement occurs during which there is an increase in the solvent-exposed hydrophobic surface. We have previously determined the structure of full-length recombinant grancalcin by X-ray crystallography in the apo state and in a partially Ca^{2+}-bound state, with one Ca^{2+} ion per grancalcin dimer (Jia *et al.*, 2000). In this structure, the N-terminal Gly/Pro-rich region is disordered. Binding of only one Ca^{2+} ion did not introduce any measurable conformational changes and left this question open. Therefore, we have continued crystallization experiments with N-terminally truncated grancalcin devoid of most of the Gly/Pro-rich region, which showed higher solubility in the presence of Ca^{2+}. We present here the structure of one such molecule, des(1–52)grancalcin, in the presence and absence of Ca^{2+}.

2. Materials and methods

2.1. Protein purification

Calcium-dependent precipitation of grancalcin was a confounding factor in our previous crystallization experiments (Han *et al.*, 2000; Jia *et al.*, 2000) and the N-terminal segment of grancalcin has been shown to be critical for calcium-dependent precipitation (Lollike *et al.*, 2001). We therefore decided to try to crystallize an N-terminal truncation mutant of grancalcin [des (1-52)grancalcin], which we knew was less prone to precipitation in the presence of high calcium concentrations (Lollike *et al.*, 2001). Des(1-52)grancalcin is named after the starting amino acid according to the nomenclature for grancalcin; thus, the initiating 52 amino acids have been removed. Des(1-52)grancalcin was cloned from the grancalcin clone using the primer 5′-CGC GGA TCC TCC GTG TAT ACT TTC AGT G-3′ for the N-terminus and the primer 5′-CCG GAA TTC TCA AAT TGC CAT AGT GCC CTG C-3′ for the C-terminus. The obtained clones and the vector (pGEX2T; Pharmacia Amersham Biotech AB) were cut with *Bam*HI and *Eco*RI and, following ligation, were transformed into *Escherichia coli*. The expressed clones were checked by sequencing (377 DNA-Sequencer, PE Biosystems, Foster City, CA, USA) and a clone with the correct sequence was used for protein expression. Procedures for expression and purification of des(1-52)grancalcin were as described for recombinant grancalcin (Han *et al.*, 2000).

Table 1
Data collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

	Apo form	Ca^{2+}-bound form
Data-collection statistics		
Space group	$P6_522$	$P2_12_12_1$
Resolution (Å)	1.9 (1.94–1.90)	1.7 (1.76–1.70)
Measured reflections	163585	145597
Unique reflections	12769	30676
Completeness (%)	99.9 (99.3)	91.1 (82.4)
R_{merge} (%)	6.1 (23.0)	7.8 (33.2)
Refinement statistics		
R factor	0.211	0.203
R_{free}	0.259	0.229
No. of residues	161	330
No. of non-H atoms	1293	2634
No. of water molecules	132	256
Average B factor	18.6	16.1
Protein (Å^2)	18.6	16.1
Water molecules (Å^2)	23.4	27.2
R.m.s.d. bond lengths (Å)	0.005	0.005
R.m.s.d. bond angles (°)	0.96	1.02
Ramachandran plot: % of residues		
In most favorable region	91.7	95.6
In disallowed regions	0.0	0.0

2.2. Crystallization and data collection

Crystallization was conducted at room temperature by the hanging-drop vapor-diffusion technique. The protein was concentrated to $\sim$8 mg ml^{-1} in a solution containing 50 mM Tris pH 7.5, 5 mM EGTA. Crystals of apo des(1-52)grancalcin were grown over a well solution containing 100 mM sodium acetate pH 4.5 and 0.7 M ammonium sulfate. These crystals display $P6_522$ space-group symmetry with unit-cell parameters $a = b = 57.6$, $c = 154.6$ Å and contain 12 molecules in the unit cell (one molecule in the asymmetric unit). Crystals of the Ca^{2+}-loaded form were obtained from wells containing 100 mM sodium cacodylate pH 6.0, 100 mM calcium acetate and 12% PEG 8000. Their space group is $P2_12_12_1$ and the unit-

cell parameters are a = 54.2, b = 71.0, c = 77.6 Å. These crystals contain two protein molecules in the asymmetric unit.

All data sets were collected at the X8C beamline at the National Synchrotron Light Source, Brookhaven National Laboratory, Upton, New York, USA. The crystals were flash-frozen to 100 K. Mineral oil was used as a cryoprotectant for the crystals of apo grancalcin and the data were recorded to 1.9 Å resolution. The crystals of the Ca^{2+}-loaded form were soaked briefly before freezing in mother liquor containing an additional 30% of glycerol; the data were collected to 1.7 Å resolution. All data processing and scaling was carried out with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Details of data collection are given in Table 1.

2.3. Structure determination and crystallographic refinement

The structures of apo and Ca^{2+}-loaded des(1-52)grancalcin were determined by the molecular-replacement method using the program *AMoRe* (Navaza, 1994). For the crystals of apo des(1-52)grancalcin, the search model consisted of one molecule of the apo full-length grancalcin dimer (PDB code 1f4q). The rotation function was calculated with X-ray data in the resolution range 20–4.0 Å and with an integration radius of 23 Å. The initial correlation coefficient was 0.278 and the crystallographic R factor was 0.512. For the crystals of Ca^{2+}-loaded des(1-52)grancalcin containing a dimer in the asymmetric unit, the search model comprised of the dimer of apo full-length grancalcin. The resolution range used for the rotation and translation functions was also 20–4.0 Å, but a slightly larger integration radius of 25 Å was used. The initial correlation coefficient was 0.389 and the crystallographic R factor was 0.489.

Model building was preformed with program *O* version 6.2 (Jones *et al.*, 1991). The program *CNS* version 0.5 (Brunger *et al.*, 1998) was used for crystallographic refinement. For cross-validation, 10% of reflections for the apo form and 5% of reflections for the Ca^{2+}-loaded form were set aside to monitor R_{free}. The structure of apo des(1-52)grancalcin has been refined to an R factor of 0.211 and R_{free} of 0.259. The final model consists of 161 residues (53–62 and 67–217) and 132 water molecules. The electron density for residues 63–66 was very poorly defined indicating disorder; they were not included in the model. The average B factor is 18.6 Å^2. The structure of Ca^{2+}-loaded des(1-52)grancalcin has been refined to an R factor of 0.203 and an R_{free} of 0.229. The final model consists of 330 residues (53–217), 256 water molecules and three Ca^{2+} ions. The average B factor is 16.1 Å^2. Both molecules have good geometry (Table 1).

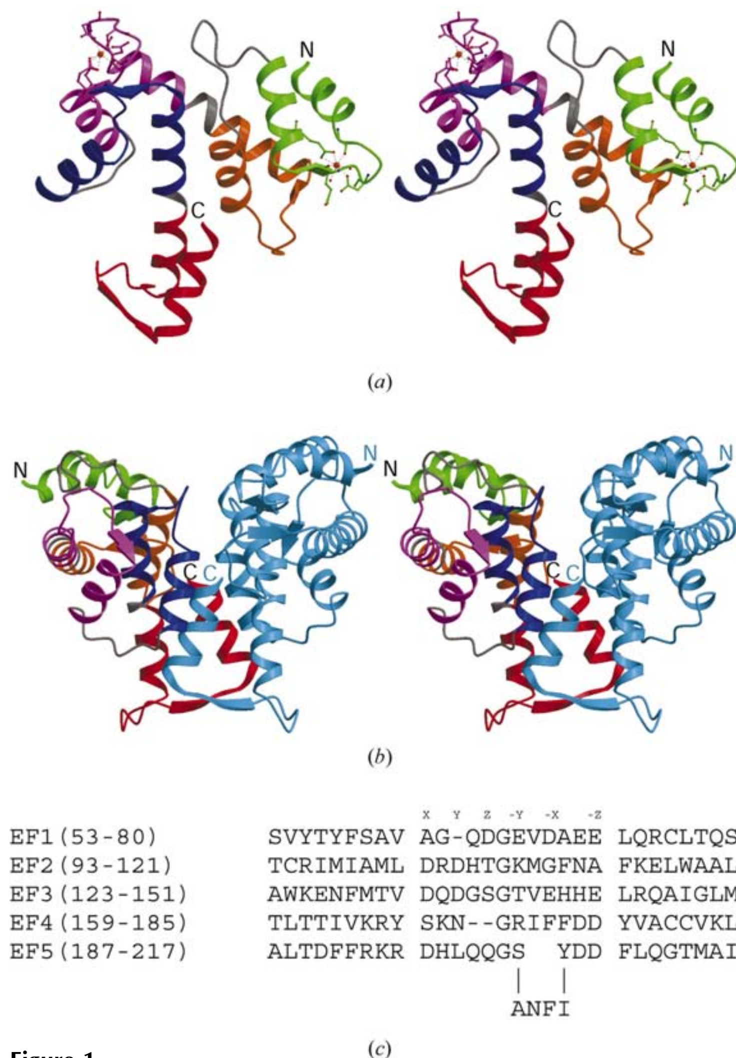

Figure 1
(*a*) Stereoview of the C^{α} trace of the des(1-52)grancalcin dimer. The side chains liganding Ca^{2+} ions are shown in full. The individual EF-hands are differentiated by color. (*b*) Cartoon drawing of the dimer. The second monomer is drawn in light blue. Figures prepared with *MOLSCRIPT* (Kraulis, 1991) and *RASTER3D* (Merritt & Bacon, 1997). (*c*) Alignment of the sequences of EF1–EF5.

3. Results and discussion

We have previously determined the structure of the full-length grancalcin crystallized in the absence and in the presence of 5 mM Ca^{2+} (Jia *et al.*, 2000). The N-terminal Gly/Pro-rich segment was disordered in both of these crystal forms and was not visible in the electron-density maps. In the crystal form grown in the presence of Ca^{2+} only one ion was found within a dimer, bound to the EF3 of one of the two molecules. The EF3-hand of the second monomer was involved in crystal contacts providing a rationale for the lack of Ca^{2+} binding to this EF-hand. None of the other EF-hands contained Ca^{2+} ions. The structure of the Ca^{2+}-containing form showed very minor differences from the apo form, with a root-mean-square (r.m.s.) deviation of 0.53 Å for all C^{α} atoms (165 C^{α}) with no conformational changes arising from Ca^{2+} binding evident in this crystal form. Attempts to obtain crystals of full-length grancalcin at a higher Ca^{2+} concentration were unsuccessful. The observed binding of Ca^{2+} to only one EF-hand within the grancalcin dimer was at variance with predictions based on sequence analysis, which indicated that the EF1-hand is competent for Ca^{2+} binding, and with flow dialysis experiments, which revealed two Ca^{2+}-binding sites per monomer (Lollike

et al., 2001). Therefore, we reasoned that the observed grancalcin dimer with only one Ca^{2+} ion bound per dimer does not represent the physiological Ca^{2+}-loaded state. The partial occupancy of EF hands by Ca^{2+} ions was most likely to be a combination of the crystal contact requirements in this particular crystal form, disorder of the N-terminal segment and the low solubility of full-length grancalcin in the presence of Ca^{2+}.

To pursue the effect of Ca^{2+} on the conformation of grancalcin, we have expressed a truncated form of grancalcin in which the 52 N-terminal residues were deleted. This des(1-52)grancalcin was significantly more soluble in the presence of Ca^{2+} than the full-length grancalcin (Lollike *et al.*, 2001). We obtained crystals of this truncated protein in its apo form and in the presence of a high concentration (100 m*M*) of Ca^{2+}.

3.1. Overall structure

The overall structure of des(1-52)grancalcin is very similar to that observed for the full-length protein (Jia *et al.*, 2000). The monomer contains five EF-hands. EF1 pairs with EF2 through a short two-stranded β-sheet and EF3 pairs in a similar way with EF4; EF5 associates with its counterpart from a second monomer, thus providing much of the surface involved in dimerization. Helices F2 and E3 are fused into one five-turn α-helix; similarly, helices F4 and E5 combine into a six-turn α-helix (Fig. 1). The two independent molecules in the crystal grown in the presence of Ca^{2+} superimpose with an r.m.s. deviation of 0.67 Å for 164 C^{α} atoms, and with an r.m.s. deviation of 1.21 Å for 159 $C\alpha$ atoms of the apo form of des(1-52)grancalcin. The superposition with the apo form of full-length grancalcin gives an r.m.s. deviation of 0.80 Å for the apo des(1-52)grancalcin and of 1.15 Å for the Ca^{2+}-loaded des(1-52)grancalcin, indicating that the overall conformation of grancalcin (excluding the N-terminus) is very similar in all crystal forms. Superposition of grancalcin molecules from all crystals forms (Fig. 2) shows however that local differences are observed, especially in the Ca^{2+}-loaded des(1-52)grancalcin, indicating limited conformational changes accompanying Ca^{2+} binding to several EF-hands.

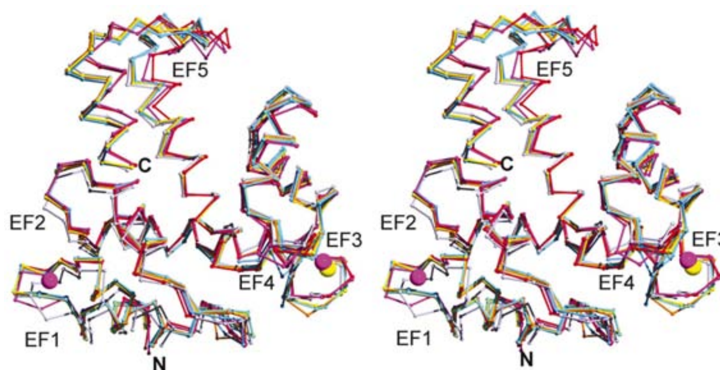

Figure 2
Superposition of independent molecules of grancalcin and des(1-52)grancalcin from all crystal forms. The molecules are color-coded: apo-grancalcin molecule *A*, cyan; molecule *B*, blue; grancalcin with Ca^{2+} molecule *A* (one Ca^{2+}), yellow; molecule *B* (no Ca^{2+}), orange; apo-des(1-52)grancalcin, blue-gray; Ca^{2+}-loaded des(1-52)grancalcin molecule *A* (two Ca^{2+}), red; molecule *B* (one Ca^{2+}), magenta. Only small differences can be noticed. The most noticeable global feature is a small displacement of EF5 in the Ca^{2+}-loaded des(1-52)grancalcin, which results in some displacement of the two EF1 hands within a dimer (not shown).

3.2. Calcium-binding sites

Biophysical data indicate that each grancalcin molecule has two Ca^{2+}-binding sites and that they most likely bind to EF1 and EF3 (Lollike *et al.*, 2001; Jia *et al.*, 2000). The structure of Ca^{2+}-loaded des(1-52)grancalcin presented here indeed confirms this prediction. We observe three Ca^{2+} ions bound to a des(1-52)grancalcin dimer; two of them are bound to the EF3-hands and the third Ca^{2+} ion binds to one of the EF1-hands. The reasons for the absence of Ca^{2+} in the second EF1-hand is clear from the crystal structure: this site is occupied by Arg154 from a symmetry-related molecule, thus preventing binding of Ca^{2+} to this EF1-hand (Fig. 3*c*). We fully expect that in solution Ca^{2+} ions bind to both EF1-hands of the dimer.

3.2.1. EF1. The Ca^{2+} binding loop in EF1 is one residue shorter than the canonical EF binding loop (Fig. 1*c*) (Kawasaki & Kretsinger, 1995; Jia *et al.*, 2000). This binding site is similar to the EF1 binding site of the Ca^{2+}-binding domain VI of calpain (Blanchard *et al.*, 1997). Pentagonal bipyramidal coordination is provided by the main-chain carbonyl group of Ala62, the side chain of Asp65, the main-chain carbonyl group of Glu67 and the side chain of Glu72 (bidentate) and two water molecules (Fig. 3*a*). Comparison of this loop in the Ca^{2+}-free state, as observed in the structures of apo grancalcins, and in the Ca^{2+}-bound state shows that the loop undergoes a rearrangement near Ala62. In the former, the carbonyl group of Ala62 points outside and its oxygen makes contact through a bridging water molecule to the main-chain O atom of Ser54. In the Ca^{2+}-bound state, the Ala62–Gly63 peptide bond is flipped and the carbonyl oxygen is now pointing toward the Ca^{2+} ion. This flipping is associated with a change of the backbone φ torsion angle of Ala62 by 180° and the φ angle of Gly63 changes to $-\varphi$ (Fig. 3*a*). The EF1 hand of the second monomer of the Ca^{2+}-loaded des(1-52)grancalcin, the center of which is occupied by the guanidino group of an arginine from a symmetry-related molecule, maintains the Ca^{2+}-free conformation. This group makes several hydrogen bonds and interacts with the side chains of Glu72, Asp65 and the main-chain carbonyl group of Asp67. We therefore conclude that the Ca^{2+} ion is necessary to provide sufficient compensation energy for a flip of the peptide bond. A comparison with the apo form of calpain domain VI shows that the conformation of this loop in the absence of Ca^{2+} differs between these proteins (Fig. 3*c*).

The flipping of the Ala62 peptide bond reorganizes the EF1 Ca^{2+}-binding loop; however, this seems to have only a small effect on the orientation of helix E1. Nevertheless, such changes may affect the disposition of the N-terminal Gly/Pro-rich peptide and amplify the Ca^{2+}-induced

Figure 3
Stereoview of EF1. (*a*) Superposition of EF1 with and without Ca^{2+}. The apo form is shown in gray. Dashed lines connect the Ca^{2+} ion with the oxygen ligands. Water molecules are marked with letter W. (*b*) Superposition of EF1 of apo grancalcin and apo calpain domain VI. Calpain is shown in gray. (*c*) EF1 in the second grancalcin monomer, with Arg154 (gray) from the neighboring molecule occupying the Ca^{2+}-binding site. (*d*) The superposition of Ca^{2+}-bound EF1 of des(1-52)grancalcin and calpain domain VI (gray) showing very similar conformations.

conformational changes. Similarly, small conformational changes were observed to be induced in calpain domain VI by the binding of Ca^{2+} (Blanchard *et al.*, 1997). A much larger rearrangement was proposed to take place in ALG-2, another protein of the PEF family (Jia *et al.*, 2001).

3.2.2. EF3. EF3 has an amino-acid sequence corresponding to a canonical Ca^{2+}-binding EF-hand (Strynadka & James, 1989; Nakayama & Kretsinger, 1994). The Ca^{2+} ion has pentagonal bipyramidal coordination and is surrounded by seven liganding O atoms, which come from the side chains of Asp132, Asp134, Ser136, the main-chain carbonyl group of Thr138, the side chain of Glu140 and the side chain of Glu143 (bidentate). This binding is somewhat different from other EF-hands from PEF family in that the protein provides

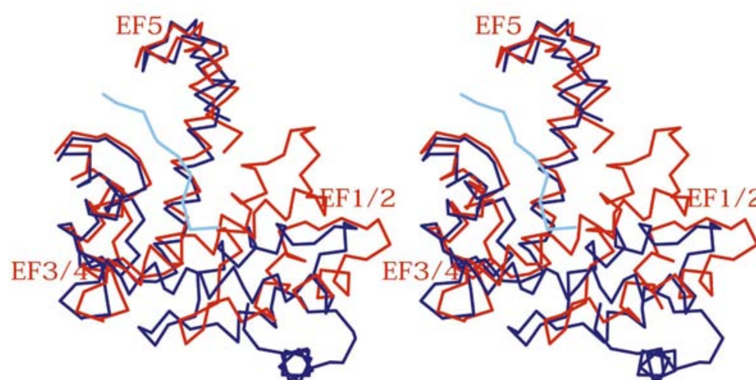

Figure 4
Superposition of Ca^{2+}-bound des(1-52)grancalcin and ALG-2. The superposition is based on EF4/5 only. Grancalcin is shown in red, ALG-2 is blue and the ALG-2-bound peptide in cyan.

all seven O atoms and no water molecule is needed to complete the coordination sphere of the ion. The axial position (marked X in the canonical EF-hand nomenclature; Fig. 1*c*), usually occupied by a water molecule, is occupied by the side-chain oxygen of Glu140. This position is occupied by a polar side chain but is not conserved among the EF-hand sequences. Within the PEF family, a serine, aspartate and glutamate are found in this position (Jia *et al.*, 2000). In ALG-2 the corresponding residue is Asp111 and this side chain is not long enough to coordinate the Ca^{2+} ion; therefore, a water molecule is sequestered. In calpain dVI, a glycine occupies this position, again requiring a water molecule as a second axial ligand (Blanchard *et al.*, 1997; Lin *et al.*, 1997). This kind of binding, in which the protein provides seven ligands to bind Ca^{2+}, is also found in the EF3 binding site of parvalbumin (Swain *et al.*, 1989).

3.3. Comparison with full-length grancalcin

The structure of grancalcin has now been determined in four crystalline environments, with and without Ca^{2+}. In all cases grancalcin exists in a dimeric form. Superposition of all independent molecules (Fig. 2) shows no significant global conformational changes induced by binding of Ca^{2+} to one or two EF-hands. The main overall effect is a slight change in the orientation of EF5, which affects somewhat the relative disposition of the molecules in the dimer, in particular the relative disposition of the two EF1-hands. Nevertheless, local changes in the EF-hand loops that bind Ca^{2+} are apparent.

We have superimposed the EF1-hands from all the molecules based on the C^{α} positions of helices E1 and F1. The conformation of the Ca^{2+}-binding loop between these two helices is rather similar in all the molecules. In particular, all four EF1-hands of full-length grancalcin are devoid of Ca^{2+} and are almost identical, with the exception of the orientation of the Gln64 side chain. The most different conformation is displayed by the EF1 of apo des(1-52)grancalcin, which is less well ordered and differs in the 63–65 segment. The Ca^{2+}-bound EF1 of des(1-52)grancalcin differs only in the main chain of Gly63–Gln64 caused by a flip of Ala62–Gly63 peptide bond, necessary for the carbonyl group of Ala62 to be able to coordinate the Ca^{2+} ion. The binding of Ca^{2+} to EF1 has neither affected significantly the E1—F1 interhelical angle nor influenced the arrangement of the EF1–EF2 pair.

To analyze the flexibility of EF3, we have locally superimposed all EF3 based on the C^{α} atoms of helices E3 and F3. The largest divergences are observed for residues 133–137 and for the side chains of residues Glu140, His141 and His142. The 133–137 segment is indeed disordered in one molecule of the apo grancalcin. Side chains of three of the residues mentioned above, Asp134, Ser136 and Glu140 (non-canonical), participate in Ca^{2+} coordination and close over the ion when it is present, while they point toward the exterior of the loop when the ion is absent. On the other hand, Glu143, which provides two oxygen ligands to the Ca^{2+} ion, does not shift significantly and in all molecules makes a hydrogen bond to the backbone NH group of Glu140, stabilizing the backbone of the C-terminal end of the EF-hand loop. The more open conformation of the EF3 loop in the absence of Ca^{2+}, and in particular the shifts of Val139 and Glu140 within the short β-sheet, affect the positions of Arg172 and Ile173 and cause a small shift of the EF4 loop.

The comparison of molecules from a variety of crystal forms obtained under different conditions shows that while the presence of Ca^{2+} affects strongly the solubility of grancalcin, these ions by themselves induce only rather small structural changes.

3.4. Comparison with calpain and ALG-2

A detailed comparison of the structure of the apo and partially Ca^{2+}-loaded form of full-length grancalcin with that of calpain domain VI has been provided in our previous paper (Jia *et al.*, 2000). The structure of des(1-52)grancalcin in the Ca^{2+}-loaded form provides further indication that Ca^{2+} binding to EF1 and EF3 reorganizes and rigidifies the loops within the EF-hands but does not lead by itself to significant conformational changes in the molecule. Indeed, the differences between Ca^{2+}-free and Ca^{2+}-loaded grancalcin are even smaller than those observed in domain VI of calpain, where a $\sim 10^{\circ}$ change in the E1—F1 interhelical angle was observed.

These observations should be related to the structure of Ca^{2+}-loaded ALG-2, which displayed a different arrangement of EF1- and EF2-hands relative to EF4- and EF5-hands (Fig. 4) (Jia *et al.*, 2001). In ALG-2 crystals, a molecule interpreted as a decapeptide was found bound to ALG-2 in addition to the Ca^{2+} ions. It was postulated that the observed difference in the orientation of the pairs of EF-hands was a combined result of Ca^{2+} and peptide binding and that the presence of Ca^{2+} ions alone would not lead to such a large conformational change. We hypothesized further that binding of a Gly/Pro-rich peptide (arising from the Gly/Pro-rich N-terminal) might be also required for conformational changes in other proteins from the PEF family. This hypothesis, which still awaits experimental confirmation, is in agreement with the observation reported here that the presence of only a high concentration of Ca^{2+} is not sufficient to induce significant structural changes in grancalcin. The hypothesis is, however, substantiated by the finding that N-terminally mutated grancalcin [like des(1-52)grancalcin] is much more soluble in the presence of Ca^{2+} ions than intact grancalcin (Lollike *et al.*, 2001).

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