The Crystal Structure of Calcium-bound Annexin Gh1 from Gossypium hirsutum and Its Implications for Membrane Binding Mechanisms of Plant Annexins

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Plant annexins show distinct differences when compared to their animal orthologues. In particular, the endonexin sequence, which is responsible for coordination of calcium ions in type II binding sites, is only partially conserved in plant annexins.

The crystal structure of calcium-bound cotton annexin Gh1 was solved at 2.5 Å resolution, and shows three metal ions coordinated in the first and fourth repeat in type II and III binding sites. While the protein has no detectable affinity for calcium in solution in the presence of phospholipid vesicles, we determined a stoichiometry of four calcium ions per protein molecule using isothermal titration calorimetry. Further analysis of the crystal structure shows that binding of a fourth calcium ion is structurally possible in the DE loop of the first repeat. Data from this study are in agreement with the canonical membrane-binding of annexins, which is facilitated by the convex surface associating with the phospholipid bilayer by a calcium bridging mechanism. In annexin Gh1, this membrane-binding state is characterised by four calcium bridges in the I/IV module of the protein, and direct interactions of several surface-exposed basic and hydrophobic residues with the phospholipid membrane.

Analysis of the protein fold stability reveals that the presence of calcium lowers the thermal stability of plant annexins. Furthermore, an additional unfolding step was detected at lower temperatures, which can be explained by the anchoring of the N-terminal domain to the C-terminal core by two conserved hydrogen bonds.

In all the kingdoms, annexins share the three-dimensional fold consisting of an N-terminal tail of variable length, and a C-terminal core which contains a 4-fold repeat (I-IV) of a 70-amino acid sequence. Structurally, each repeat consists of a four-helix bundle (A, B, D, E) and a fifth helix (C) almost perpendicular to the bundle underneath. Many biochemical and histological properties are shared among all annexins due to this structural conservation, although differences have also been observed (1). The calcium-dependent binding to acidic phospholipid membranes has been a landmark feature of annexins, and this is due to the presence of canonical calcium binding sites provided by the endonexin sequence K-G-X-G-T-{38}-D/E (2). In animal annexins, this motif is present in three or four repeats of the C-terminal core and localises to the convex and membrane-binding side of the slightly curved protein.

Although plant annexins have been implicated in a variety of physiological processes, assignment of individual and detailed roles await further elucidation. An involvement of annexin D1 from Arabidopsis thaliana in oxidative stress response has been reported (3), and it was hypothesised for cotton annexin Gh1 (4). In Medicago sativa, annexin Ms2 is up-regulated upon environmental stress, such as drought and osmotic stress (5). For other plant annexins, roles in mechanical stress response (Bryonia dioica) (6), low temperature signal transduction (Lavatera thuringiaca and Triticum aestivum) (7; 8), cell volume and vacuole size regulation (Nicotiana tabacum) (9), as well as exocytosis (Zea mays) (10) have been proposed. Using Arabidopsis seedlings, a role of annexins in differential growth during gravitropism has been implied (11). Several observations further suggest
a link between plant annexins and polysaccharide synthesis, an important factor of the plant’s response to infection and wounding. Annexins from *Lilium longiflorum* and *Arabidopsis thaliana* have been involved in Golgi-mediated secretion of polysaccharides (12; 13). Cotton annexins have been found to co-purify with calllose [(1→3)-β-D-glucan] synthase (14-16), and an inhibitory effect of cotton annexins on polysaccharide synthesis has been observed (14). The discovery of an unusual sulphur cluster in the crystal structure of annexin Gh1, and its conservation in many plant annexins, fuelled the hypothesis of a RedOx regulation of cellulose synthase by the annexin (4; 17; 18). The recent discovery of an oomycete annexin that acts as an activator of calllose synthase adds further weight to this notion (19).

Ever since plant annexins were introduced to the wider annexin family (20; 21), the variation or absence of the endonexin sequence in some repeats was evident from comparison of the primary structures (12; 22; 23). While calcium-dependent binding to phospholipid membranes has been reported for all plant annexins investigated so far (7; 20; 24-28), canonical calcium binding in plant annexins has only been predicted for repeats I and IV (28-30). Furthermore, the existence of calcium-dependent and calcium-independent protein-membrane interactions have been observed with bell pepper and cotton annexins, and three conserved surface-exposed residues on the convex side of the molecule have been identified as regulators of the calcium-independent membrane binding (31).

Although crystal structures of plant annexins from *Capsicum annuum*, *Gossypium hirsutum*, and *Arabidopsis thaliana* have been determined, none were obtained in the presence of calcium. Therefore, it remains unclear which calcium binding sites are utilised by plant annexins. The endonexin sequence constitutes the type II calcium binding sites, where the coordination sphere of calcium typically is a pentagonal bipyramid with a backbone carbonyl oxygen and a water molecule in apical positions. Another water molecule, three backbone carbonyl groups, and the acidic residue from the conserved motif form the base of the bipyramid. In contrast, type III and AB’ sites involve one or two backbone carbonyl groups and a bidentate acidic residue nearby. The coordination sphere of calcium in these sites is completed by several water molecules. The type III binding sites are observed in the DE loops of the annexin repeats. In annexin B12, intermolecular calcium coordination bridging two annexin monomers in a head-to-head arrangement has been observed (32). Recently, the calcium-bound structure of alpha-11 giardin from *Giardia lamblia* revealed a new binding site (type IIIb) in the DE loop of repeat I, where the metal ion is coordinated by three backbone carbonyl oxygen atoms of Lys53, Ile56, and Lys57, as well as side chain oxygen atoms from Asn58 and Glu62 (mono-dentate) (33). While in three of the four molecules in the asymmetric unit, the calcium is coordinated as an intermolecular ligand, one monomer possesses an intramolecular calcium where two water molecules complete the coordination sphere of a pentagonal bipyramid.

In this study, we determined the crystal structure of cotton annexin Gh1 in the presence of calcium and provide, for the first time, insights into the structural constituents of plant annexin calcium binding. Using isothermal titration calorimetry, the calcium stoichiometry of annexin Gh1 in the membrane-bound state was determined. Thermal denaturation experiments reveal the destabilisation of cotton and bell pepper annexins in the presence of calcium in solution, an observation that is highly unusual.

**EXPERIMENTAL PROCEDURES**

*Expression and purification of recombinant protein.* The wild-type and fusion proteins used in this study were cloned and purified as described previously (25; 31; 34).

*Crystallisation.* Native crystals of recombinant annexin Gh1 were obtained by the hanging drop vapour diffusion method using a reservoir solution of 1.7 M (NH₄)₂SO₄, 0.1 M HEPES (pH 7.0) (4). Droplets consisted of 2 µl protein solution (25 mg/ml) and 2 µl reservoir solution. Crystals were obtained after about four weeks at 290 K. Initially, soaking and co-crystallisation of native crystals were attempted in the presence of 1 – 15 mM CaCl₂. A fine grid search around these conditions was conducted by adjusting the concentrations of the precipitant, as well as the buffer pH. Due to the formation of insoluble calcium sulphate at calcium concentrations of more than 30 mM, subsequent co-crystallisation trials with higher concentrations of calcium (50 mM and 100 mM) were carried out with in-house screening buffers containing no ammonium sulphate. Crystals of calcium-bound annexin Gh1 were obtained from
1.6 M KH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 6.0) and 50 mM CaCl$_2$.

**Data collection, structure solution, and refinement.** Crystals were cryo-protected with 25% glycerol, and diffraction data was collected at 100 K at beam line BM14 of the European Synchrotron Radiation Facility (Grenoble, France). Data sets were indexed using the program MOSFLM (35) version 6.2.3. Intensities were scaled and merged with SCALA from the CCP4 package (36). Unit cell parameters and data collection statistics are shown in Table 2. The structures were solved by molecular replacement using the program MOLREP (37) and the atomic coordinates of the apo-protein (PDB accession number 1n00) (4). Residues in the loop regions were substituted for alanine for the Patterson search. Structure refinement with REFMAC (38) used initial rigid body refinement, and subsequent rounds of positional, grouped and individual B-factor refinement. A constant bulk solvent model was applied throughout the procedure. 5% of the data were used to calculate an $R_{	ext{free}}$-factor. The initial model was visually inspected and manually corrected with COOT (39). Subsequently, the loop regions were rebuilt unambiguously using omit electron density maps. The final geometry was assessed with PROCHECK (40). For a summary of refinement statistics see Table 2. Coordinates and structure factors of GH1_26 have been deposited with the PDB under accession number 3BRX.

**Preparation of phospholipid vesicles.** Small uni-lamellar vesicles (SUVs) with diameters ranging from 15 – 50 nm were prepared by dispersion of multi-lamellar vesicles (MLVs) using sonication. The preparation procedure follows that of Pagano and Weinstein with some modifications (41). Appropriate amounts of DMPC and DMPS (mol 3:1) were transferred to a round-bottom test tube and dissolved thoroughly in chloroform/methanol (2:1 v/v). A stream of nitrogen was applied to evaporate the organic solvent. The resulting thin lipid film was then placed in a vacuum for 12 h to eliminate residual traces of organic solvent. Aqueous buffer was then added to the test tube to a final concentration of 10 mg/ml phospholipids, and vortexed at 35°C to free the lipid molecules from the test tube, thus producing a milky multi-lamellar vesicle solution. Subsequently, bath sonication was performed to disperse the MLVs and convert them to SUVs. A gentle nitrogen stream was also employed to avoid oxidative degradation of the lipids during sonication. The temperature of the sonication bath was kept at 4°C during the whole process. The preparation of SUVs was complete when the lipid solution adopted a clear, slightly blue colour.

**Isothermal titration calorimetry.** Calcium binding of annexin Gh1 in the absence and presence of DMPC/DMPS (molar ratio 3:1) was assessed using an isothermal titration calorimeter VP-ITC from MicroCal. All solutions were degassed. Samples were placed into the calorimetry cell (volume: 1.4 ml), and the titration syringe contained a solution of 50 mM CaCl$_2$ in the absence, and 5 mM CaCl$_2$ in the presence, of vesicles respectively. All samples were prepared in a buffer containing 100 mM NaCl and 20 mM TRIS (pH 8.0). The concentration of annexin Gh1 was 75 µM in the absence, and 25 µM in the presence, of lipid vesicles. For experiments with vesicles, the final amount of lipids was 7.5 mg/ml. The experiments were performed at 25°C by titration of 5 µl portions. For every titration a reference experiment was carried out by titrating calcium into a sample without protein. Analysis was performed offline using the program Origin (MicroCal) with a special module for ITC data treatment. The heat release was calculated by integrating each titration spike. The difference of heat release between the regular and reference experiments was plotted against the molar ratio $x = n$(Ca$^{2+})/n$(protein).

**Circular dichroism spectroscopy.** Thermal denaturation of proteins was monitored by CD spectroscopy using a JASCO J-810 spectropolarimeter equipped with a Peltier element. CD spectra of a sample of 3 µM protein in 20 mM NaCl, 5 mM HEPES (pH 8.0) were recorded at different temperatures in the absence and presence of calcium (10 mM). Before each run, the sample was equilibrated at the respective temperature for 20 min. The effect of calcium on the secondary structure was assessed by collecting CD spectra at 20°C and different calcium concentrations. All experiments were carried out three times independently. Using the software program ACDP (42; 43), each protein spectrum was corrected with a buffer spectrum acquired at the same temperature, and then transformed into mean residue ellipticity. Changes in the mean residue ellipticity at 222 nm were used to construct an unfolding curve. Curve fitting of an appropriate range of data was done.
using a sigmoid equation in SigmaPlot (SPSS, Inc.).

RESULTS

Crystal structure of calcium-bound annexin Gh1. Determination of the calcium-bound cotton annexin Gh1 crystal structure was only possible by exchanging the major precipitant from sulphate to phosphate salts and rather high calcium concentrations. While soaking the protein crystals with high calcium concentrations cracked the crystals and rendered them unusable, co-crystallisation at high levels of calcium required precipitants other than sulphate salts.

At low calcium concentrations, the crystal shape is triangular prism-like, and the size of crystals decreased with increasing calcium concentrations (see Figure 1). Crystals of annexin Gh1 obtained from phosphate conditions in the presence of 50 mM calcium appear as long rods. This apparent change in the crystal shape is reflected by the change in space group from P3121 to P321, and the change in unit cell dimensions in data set GH1_26. The crystallographic 3-fold symmetry leads to three annexin molecules packed tightly in the same plane with the contact interfaces provided by the IAB and IIAB loops of symmetry-related molecules. The crystallographic 2-fold axis creates an interaction interface formed by helices IID, IIE, and IIIA.

In data set GH1_26, three peaks in the electron density indicate the presence of calcium ions (see Figure 2 and Table 1). In the IAB loop, a calcium ion (CA1) is coordinated with pentagonal bipyramidal geometry. Three carbonyl oxygen atoms (Phe29, Gly31, Gly33), the bidentate carboxylate of Glu73, and a water molecule provide the coordination of a type II binding site as anticipated earlier (30). The apical ligand of CA1 is provided by the carbonyl oxygen of Trp104 from the IIAB loop of a symmetry-related molecule. The side chain of Trp104 faces a pocket of the neighbouring molecule, indicating hydrogen bonding. The conjugated \( \pi \)-electron system of Trp32 is in stacking conformation with Arg80 from the top part of the IE helix of a neighbouring molecule, indicating hydrogen bonding. The side chain of Trp104 faces a pocket provided by Lys66, Ala69, Lys72, and Glu73 from another symmetry-related molecule.

Isothermal titration calorimetry. In the absence of phospholipid vesicles, no significant enthalpy change was detected when titrating calcium into a sample of annexin Gh1 over a wide range of molar ratios (Figure 4). This agrees with the CD spectra analysis of annexin Gh1 at various calcium concentrations (0 to 9 mM) where no significant changes in the signal at 222 nm are observed (data not shown). Thus, there is

Superposition of the current structure with other structures of apo-plant annexins shows the usual conformational divergence with rms deviations of 2.4 – 3.0 Å. Compared to the structure of apo-annexin Gh1 (rms deviation: 1.3 Å), the only significant conformational differences occur in loops IAB, IDE, IIAB, IVAB, and IVDE (see Figure 3). Loop IVAB moves closer towards loop IVDE, with Thr264 relocating by 9 Å. The second most significant movement is observed in loop IIAB, where Trp104 moves about 4 Å, and the guanidinyl group of Arg103 by about 7 Å. The semi-sheltered position of Trp104 in the apo-structure where Arg103 shields the tryptophan residue, has changed to fully expose Trp104 and Arg103. The carbonyl oxygen of Trp104 relocates by 8 Å to participate in the coordination of a calcium ion in the first repeat of a symmetry-related molecule. Within the first repeat, less re-arrangement is observed upon calcium binding. Glu73 moves downwards and the backbone of Trp32 towards the IDE loop, each by 2 Å. Occupation of the type III binding site in the IVDE loop leads to a slight re-positioning of CO-301 and CO-304 by 2.5 and 1.5 Å, respectively.

Neither of the two exposed tryptophan residues is harboured in a predominantly hydrophobic pocket. The conjugated \( \pi \)-electron system of Trp32 is in stacking conformation with Arg80 from the top part of the IE helix of a neighbouring molecule, indicating hydrogen bonding. The side chain of Trp104 faces a pocket presented by Lys66, Ala69, Lys72, and Glu73 from another symmetry-related molecule.

...
no association of calcium ions with cotton annexin Gh1 in solution. A similar phenomenon is well known for several animal annexins which show only low affinity for calcium in the absence of phospholipid membranes (48; 49). However, in the presence of DMPC/DMPS (3:1) vesicles, annexin Gh1 titration with calcium exhibited downward spikes, indicating an exothermic reaction after each titration event. A plot of the heat release as a function of the molar ratio revealed a graph that could be fit with a model of one set of binding sites (Figure 4). The fit parameters reveal a stoichiometry of 4.35 ± 0.154 mol of calcium per 1 mol of annexin Gh1. The apparent dissociation constant for the ternary complex annexin Gh1:Ca$^{2+}$:membrane is $K_d = 11 \mu M$. This results in a c-value of the ITC profile of 9.08, which is near the lower edge of the experimental window.

**Stability measurements.** The stability of bell pepper and cotton annexins was assessed by thermal denaturation studies. The CD signal at 222 nm was used to monitor changes in secondary structure upon unfolding of the protein (see Figure 5 and summarised results in Table 3). Surprisingly, two transitions were observed for the plant annexins tested in this study.

The first transition is at about 28°C, and is not dependent on the presence of calcium. It is not entirely clear what the reason for this additional transition is since the elongation of the N-terminal domain by the poly-His-fusion is not the cause. The transition might be due to some conformational change in the original N-terminal domain, or a more global conformational change in the protein core.

The second transition corresponds to the main unfolding of the protein secondary structure and happens, in the absence of calcium, at $T_{1/2} = 63°C$ and 58°C for the native and the hexa-His-tagged bell pepper annexin. Both proteins are thus more stable than annexin A5. The tetra-His-fusion construct of cotton annexin Gh1 is slightly less stable than annexin A5 ($\Delta T_{1/2} = -2 K$). In the presence of 10 mM calcium, all plant annexin proteins tested are destabilised as indicated by the lower transition temperatures for the secondary structure unfolding. The temperature difference is -8 K and -5 K for wild-type and fusion bell pepper annexin, respectively; for cotton annexin Gh1, the temperature difference is -10 K. This phenomenon is in sharp contrast to the situation found with mammalian annexins, such as AnxA5 (50), which are usually stabilised in the presence of calcium.

**DISCUSSION**

The structure of calcium-bound cotton annexin reveals the conserved annexin fold with three calcium ions bound in a canonical fashion in repeats I and IV, as previously predicted (30). Analysis of the conformations in both the apo- and calcium-bound crystal structures indicate that binding of calcium in type II or type III sites in repeats II and III does not seem possible.

**Conformational flexibility of loop residues in plant annexins.** When comparing calcium-bound annexin Gh1 to the earlier crystal structures of cotton and bell pepper annexins (the structure of annexin D1 does not contain a model of the IAB loop region), the conformational flexibility of the IAB loop, in particular Trp35, is striking (see Figure 6). In an earlier study, we have shown that the highly conserved tryptophan in the first repeat is an important determinant of plant annexin membrane binding by contributing to the calcium-independent binding mode (31). One can hypothesise that by sampling different conformations with this residue, the plant proteins can rapidly associate with a phospholipid membrane surface which is likely to be the first step in the membrane binding process. A similar role can be envisioned for Trp104, which has also been shown to play a pivotal role in calcium-independent membrane association of plant annexins.

As expected, the crystal structure of calcium-bound cotton annexin Gh1 emphasises the flexibility in the overall structure of the calcium-binding loop areas IAB/IDE and IVDE on the convex side of the protein. The conformational changes in the first repeat are mainly observed with side chain conformations. No major rearrangements are necessary, because apo-annexin Gh1 adopts a conformation that enables coordination of a calcium ion (4). The significant conformational change of backbone conformation in repeat IV was also anticipated (4), and brings the AB loop in closer position to the DE loop, thereby establishing the appropriate coordination environment for the metal ion. Such large movements are not unprecedented in annexins. The best studied one is certainly the calcium-triggered conformational change of the IIIAB loop in annexin A5. Surprisingly, while domain II does not harbour a functional calcium binding site, IIIAB residues are involved in coordination of a calcium ion in the present structure. The second most significant change in
backbone conformation in the present structure is observed in the IIAB loop. While Arg103 and Trp104 adopted semi-sheltered positions in the apo structure, both residues are now fully exposed and probably directly interacting with phospholipid molecules in the membrane-bound state. This finding is in agreement with our previous results (31). The unfavourable exposure of Trp104 to solvent in the absence of a membrane is compensated by the crystal packing. In the present structure Trp104 contributes to coordination of a calcium ion in the IAB loop of a symmetry-related molecule which energetically stabilises the exposed conformation.

(calcium binding, and implications for the membrane-bound state. Calcium titration of annexin Gh1 in solution using isothermal titration calorimetry reveals no detectable binding in the molar ratio range of up to 350. The CD spectra of annexin Gh1 at molar ratios of protein and calcium in the same range confirm this finding, since no calcium-correlated change of the signal is observed.

Similar to their animal relatives, plant annexins show a significantly increased affinity for calcium in the presence of acidic phospholipid vesicles. For cotton annexin Gh1, our results from isothermal titration calorimetry yield a molar ratio of 4 calcium ions per protein molecule in the membrane-bound state.

While only three calcium ions have been located in the present crystal structure, analysis of the IDE loop region of the calcium-bound crystal structure of annexin Gh1 shows that a water molecule is occupying the putative type III binding site of the first repeat. The backbone carbonyl oxygen atoms of Asp71 (2.56 Å) and Leu74 (2.58 Å), as well as the bidentate carboxylate group of Glu79 (OE1: 3.44 Å, OE2: 2.75 Å) adopt a conformation that would enable coordination of a calcium ion instead of water molecule HOH115. Other possible contributing ligands include the carboxyl group of Asp71 as mono-dentate ligand (OD2: 3.27 Å), and another water molecule, HOH123 (2.84 Å). In the present structure, the conformations seen for the coordination of the three calcium ions would allow for the canonical binding to a phospholipid membrane by simply exchanging a few coordinating ligands of the metal ions with phospholipid groups. We therefore hypothesise that the canonical membrane-bound species of cotton annexin Gh1 utilises the type II and type III calcium binding sites in repeats I and IV for the calcium-dependent binding mode. Plant annexins therefore seem to bind a maximum of four calcium ions in a canonical fashion on the I/IV module. This finding is in agreement with a mutagenesis study of tomato annexin Le35 (28). Furthermore, such an arrangement would require the direct interaction of Trp32 and Trp104 with the phospholipid head group or glycerol backbone of the lipid membrane. The conformations found for other surface residues indicate that Arg103, Lys187, Lys230, and Arg262 will also engage in direct contact with the membrane.

Protein fold stability. Thermal denaturation experiments of the three plant annexin constructs in this study reveal two transitions with CD-monitoring. A three-step unfolding process has been observed earlier with annexin A3 (51) where it was concluded that the N-terminal domain presents an independent folding unit, since a tryptophan residue anchored the N-terminal domain to the protein core – a special feature of that particular annexin. Interestingly, two highly conserved residues in plant annexins are Ser/Thr3 and His40 (annexin Gh1 numbering). Analysis of the known plant annexin structures shows that His40 is hydrogen-bonded to the backbone carbonyl of residue 5, and the side chain hydroxyl group of Ser3 or Thr3 maintains a hydrogen bond with the backbone carbonyl of residue 311. This anchors the N-terminal domain to the protein core and may explain the low-temperature unfolding step visible in the CD-monitored denaturation. The fact that this anchoring is observed in the apo- and calcium-bound crystal structures of plant annexins agrees with the finding that the transition in the thermal denaturation is not dependent on the presence of calcium. It can thus be concluded that the coordination of calcium in the loop areas of the C-terminal core and the accompanying conformational changes do not affect the anchoring of the N-terminal to the C-terminal domain. Additionally, the observation of this phenomenon in different plant annexins and the conservation of the residues involved imply that this might be a common feature of plant annexins. This is also a distinct difference to annexins from other kingdoms, where the N-terminal domain of individual proteins is unique in their primary, secondary and tertiary structures. Further experiments will be needed to reveal the functional significance of this feature.

In contrast to mammalian annexins, all plant annexins tested in this study are significantly less stable in the presence of calcium. Based on the
ITC experiments with cotton annexin Gh1, one can conclude that there is no calcium binding in solution. Furthermore, a significant calcium-dependent effect on the oligomerization state of plant annexins has not been observed (34). This implies that calcium interacts with an unfolded state of the protein. The destabilising effect of calcium might thus be explained by a stabilisation of the unfolded state of the protein by the metal.

In this context, it is noteworthy that the poly-His-fusion at the N-terminal domain destabilises the annexin in thermal denaturation with respect to the secondary structure unfolding (2nd transition), as seen by the difference in ΔT_{1/2} of -5 K for the wild type and the His-tagged annexin 24(Ca32), respectively.

CONCLUSION

The property of calcium-dependent membrane binding of plant annexins is well-established. Recently, we have shown that these proteins possess a second membrane binding mode which is independent of calcium and involves exposed hydrophobic and basic surface residues. The current study reveals insights into the mode of calcium binding of cotton annexin Gh1 and the implications for the membrane-bound state of the protein. In the crystal structure, we could locate three calcium ions in type II and type III binding sites in repeats I and IV. As observed in earlier crystal structures, the conformations in repeats II and III do not allow for the coordination of calcium ions. The conformation found in the IDE loop region would allow binding of a fourth calcium ion. This is a highly likely possibility, since we have determined a stoichiometry of 4 calcium ions per protein molecule in the membrane-bound state of annexin Gh1.

The high similarity of cotton annexin Gh1 with other plant annexins of the Sp32 group (52), makes these findings applicable to other plant annexins, such as bell pepper annexin 24(Ca32). The location of calcium ions in the protein and the discovery of the conserved anchoring mechanism of the N-terminal domain are important molecular mechanisms that will help determine the workings of plant annexins in cellular processes.

REFERENCES


FOOTNOTES

We thank the staff at ESRF (Grenoble) for on-site support, and Ian McNae for collecting data set GH1_26. AH gratefully acknowledges financial support of this study by the Nuffield Foundation, UK (Award to newly appointed Lecturers NAL/00629/G).

FIGURE LEGENDS

Fig. 1. Co-crystallisation of annexin Gh1 with varying concentrations of CaCl₂. 1 mM (A), 5 mM (B), 10 mM (C), 15 mM (D), 50 mM (E). The crystallisation solution is based on (NH₄)₂SO₄ for A-D, and on KH₂PO₄/Na₂HPO₄ for E. At 50 mM CaCl₂, we also observed formation of salt crystals as evident by the large compact crystals in E.

Fig. 2. Coordination of the three calcium ions in annexin Gh1. (A) Type II binding site in IAB loop, (B) type II binding site in IVAB loop, (C) type III binding site in IVDE loop. Calcium ions are shown as yellow spheres and water molecules as blue spheres. The 2Fₒ-Fc electron density is contoured at 1.7σ. Figure prepared with PyMOL (53).

Fig. 3. Superposition of apo- (blue) and calcium-bound (red) annexin Gh1 as Cα plot. Upper panel: standard view from the front; lower panel: top view onto membrane binding surface. Figure prepared with PyMOL (53).
Fig. 4. Isothermal calorimetric titration profiles of His$_4$-annexin (Gh1) with CaCl$_2$ in the absence of SUVs (A), and presence of SUVs (B). (A) 25 µM of His$_4$-annexin (Gh1) titrated with 50 mM Ca$^{2+}$. (B) 25 µM of His$_4$-annexin (Gh1) titrated with 5 mM Ca$^{2+}$ in the presence of DMPC/DMPS (mol 3:1) SUVs. The solid line represents the fit to a model of one set of binding sites with a stoichiometry of 4.35 mol Ca$^{2+}$ per 1 mol protein. The upper panels show the time-dependent heat release during sample and control injections. The lower panels show the integrated heat release for each injection after baseline correction.

Fig. 5. Thermal denaturation of plant annexins as monitored by their CD at 222 nm. A Annexin 24(Ca32), B His$_6$-Annexin 24(Ca32), C His$_4$-Annexin (Gh1). Shown are the averages of at least three independent measurements in the absence (open circles) and presence of 10 mM calcium (closed circles). The solid (with calcium) and dashed (without calcium) lines represent sigmoid regression fits in appropriate areas of the graphs.

Fig. 6. The conformational switch of the IAB loop in plant annexins. Calcium-bound cotton annexin Gh1 shown in red, apo-annexin Gh1 in brown, the two conformations of bell pepper annexin 24(Ca32) shown in green. Figure prepared with Molscript (54) and rendered with Raster3d (55).
### Table 1. Calcium coordination geometry

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<td>CA1 IAB</td>
<td>II</td>
<td>7</td>
<td>Phe29: 2.37; Gly31: 2.58; Gly33: 2.53; Trp104*: 2.41</td>
<td>Glu73: 3.04 (OE1), 2.71 (OE2)</td>
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<td>Asp303: 2.44 (OD1), 2.78 (OD2)</td>
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<td>CA3 IVDE</td>
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<td>Val301: 2.15; Thr304: 2.67</td>
<td>Glu309: 2.91 (OE1), 3.00 (OE2)</td>
<td>HOH96: 2.96; HOH134: 2.38; PO₃: 2.50 (O4)</td>
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Values in the last three columns show the coordination distance to calcium in Å.
Table 2. Data collection and refinement statistics

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<td>$c$(Ca$^{2+}$) (mM)</td>
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**Data collection**

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<td>No of independent reflections</td>
<td>21804</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>5.5 (5.5)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>$R_{merge}^{a}$</td>
<td>0.117 (0.397)</td>
</tr>
</tbody>
</table>

**Refinement**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No of reflections in working set</td>
<td>20672 (1522)</td>
</tr>
<tr>
<td>No of reflections in test set</td>
<td>1116 (85)</td>
</tr>
<tr>
<td>No of non-H protein atoms</td>
<td>2832</td>
</tr>
<tr>
<td>No of water molecules</td>
<td>261</td>
</tr>
<tr>
<td>No of calcium ions</td>
<td>3</td>
</tr>
<tr>
<td>No of phosphate ions</td>
<td>5</td>
</tr>
<tr>
<td>Average B-factor (Å$^2$)</td>
<td>25.3</td>
</tr>
<tr>
<td>Ramachandran plot (%)$^b$</td>
<td>90.5 / 8.8 / 0.7 / 0</td>
</tr>
<tr>
<td>rmsd B-factor for bonded atoms (Å$^2$)</td>
<td>1.231</td>
</tr>
<tr>
<td>rmsd bond lengths (Å)</td>
<td>0.011</td>
</tr>
<tr>
<td>rmsd bond angles (°)</td>
<td>1.641</td>
</tr>
<tr>
<td>$R^c$</td>
<td>0.193 (0.267)</td>
</tr>
<tr>
<td>$R_{free}^{d}$</td>
<td>0.252 (0.319)</td>
</tr>
</tbody>
</table>
Values for the last resolution shell are given in parentheses.

\[ R_{\text{merge}} = \frac{\sum |I - \langle I \rangle|}{\sum I} \], where \( I \) is the observed intensity, and \( \langle I \rangle \) is the average intensity obtained from multiple observations of symmetry-related reflections after rejections.

\( \text{Residues in most favoured / additionally allowed / generously allowed / disallowed region.} \)

\[ R = \frac{\sum |F_o - F_c|}{\sum |F_o|} \], where \( F_o \) and \( F_c \) are the observed and calculated structure factors, respectively.

\( R_{\text{free}} \) defined in (56).
Table 3. Thermal denaturation

1\textsuperscript{st} transition

<table>
<thead>
<tr>
<th>Protein</th>
<th>T\textsubscript{1/2} in °C</th>
<th>Δ(T\textsubscript{1/2}) in K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Ca\textsuperscript{2+}</td>
<td>10 mM Ca\textsuperscript{2+}</td>
</tr>
<tr>
<td>annexin 24(Ca32)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>His\textsubscript{6}-annexin 24(Ca32)</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>His\textsubscript{4}-annexin Gh1</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>annexin A5\textsuperscript{a}</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2\textsuperscript{nd} transition

<table>
<thead>
<tr>
<th>Protein</th>
<th>T\textsubscript{1/2} in °C</th>
<th>Δ(T\textsubscript{1/2}) in K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Ca\textsuperscript{2+}</td>
<td>10 mM Ca\textsuperscript{2+}</td>
</tr>
<tr>
<td>annexin 24(Ca32)</td>
<td>63</td>
<td>55</td>
</tr>
<tr>
<td>His\textsubscript{6}-annexin 24(Ca32)</td>
<td>58</td>
<td>53</td>
</tr>
<tr>
<td>His\textsubscript{4}-annexin Gh1</td>
<td>48</td>
<td>38</td>
</tr>
<tr>
<td>annexin A5\textsuperscript{a}</td>
<td>52</td>
<td>59</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The values for human annexin A5 are given for comparison. Data from (50).
Figure 4

A

B
Figure 5

A

Degree of unfolding vs. Temperature (°C) for Annexin 24(Ca32).

B

Degree of unfolding vs. Temperature (°C) for His<sub>6</sub>-Annexin 24(Ca32).

C

Degree of unfolding vs. Temperature (°C) for His<sub>4</sub>-Annexin (Gh1).