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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Plants annexins show distinct differences in comparison with 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 types II and III binding sites. Although 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 showed that binding of a fourth calcium ion is structurally possible in the DE loop of the first repeat. Data from this study agree 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 characterized by four calcium bridges in the I/IV module of the protein and by direct interactions of several surface-exposed basic and hydrophobic residues with the phospholipid membrane. Analysis of the protein fold stability revealed 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 kingdoms, annexins share the three-dimensional fold consisting of an N-terminal tail of variable length and a C-terminal core that 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 (helix C) almost perpendicular to the bundle underneath. Many biochemical and histological properties are shared among all annexins because of 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, due to the presence of canonical calcium binding sites provided by the endonexin sequence KGXGT38(D/E) (2). In animal annexins, this motif is present in three or four repeats of the C-terminal core and localizes to the convex and membrane-binding side of the slightly curved protein.

Although plant annexins have been implicated in a variety of physiological processes, the assignment of individual and detailed roles awaits further elucidation. The involvement of annexin D1 from Arabidopsis thaliana in oxidative stress response has been reported (3) and has been hypothesized 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 aestivalum) (7, 8), and cell volume and vacuole size regulation (Nicotiana tabacum) (9), as well as exocytosis (Zea mays) (10), have been proposed. Using Arabidopsis seedlings, a role for 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 response to infection and wounding. Annexins from Lilium longiflorum and A. thaliana are involved in Golgi-mediated secretion of polysaccharides (12, 13). Cotton annexins have been found to co-purify with callose synthase (1,3-β-glucan synthase) (14–16), and an inhibitory effect of cotton annexins on polysaccharide synthesis has been observed (14). The discovery of an unusual sulfur cluster in the crystal structure of annexin Gh1, and its conservation in many plant annexins, fueled 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 callose 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 has been evident from the comparison of the primary structures (12, 22, 23). Although calcium-dependent binding to phospholipid membranes has been reported for all plant annexins investigated thus far (7, 20, 24–28), canonical calcium binding in plant annexins has been predicted only for repeats I and IV (28–30). Furthermore, the existence of calcium-dependent and -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 *A. thaliana* have been determined, none were obtained in the presence of calcium. Therefore, it remains unclear which calcium binding sites are utilized 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 the 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 with 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 α-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 Lys-53, Ile-56, and Lys-57, as well as side chain oxygen atoms from Asn-58 and Glu-62 (mono-dentate) (33). Although in three of the four molecules in the asymmetric unit, the calcium is coordinated as an intermolecular ligand, one monomer possesses an intramolecular calcium in which two water molecules complete the coordination sphere of a pentagonal bipyramid.

In this study, we have determined the crystal structure of cotton annexin Gh1 in the presence of calcium, providing, for the first time, insights into the structural constituents of plant annexin calcium binding. Using isothermal titration calorimetry, we determined the calcium stoichiometry of annexin Gh1 in the membrane-bound state. Thermal denaturation experiments revealed the destabilization 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).

**Crystallization**—Native crystals of recombinant annexin Gh1 were obtained by the hanging drop vapor diffusion method using a reservoir solution of 1.7 M (NH₄)₂SO₄ and 0.1 M HEPES (pH 7.0) (4). Droplets consisted of 2 μl of protein solution (25 mg/ml) and 2 μl of reservoir solution. Crystals were obtained after about 4 weeks at 290 K. Initially, soaking and co-crystallization 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. Because of the formation of insoluble calcium sulfate at calcium concentrations of more than 30 mM, subsequent co-crystallization trials with higher concentrations of calcium (50 and 100 mM) were carried out with in-house screening buffers containing no ammonium sulfate. Crystals of calcium-bound annexin Gh1 were obtained from 1.6 M KH₂PO₄/Na₂HPO₄ (pH 6.0) and 50 mM CaCl₂.

**Data Collection, Structure Solution, and Refinement**—Crystals were cryoprotected with 25% glycerol, and diffraction data were collected at 100 K at beam line BM14 of the European Synchrotron Radiation Facility (Grenoble, France). Data sets were indexed using the program MOSFLM, version 6.2.3 (35). Intensities were scaled and merged with SCALA from the CCP4 package (36). Unit cell parameters and data collection statistics are shown in Table 1. The structures were solved by molecular replacement using the program MOLREP (37) and the atomic coordinates of the apo-protein (Protein Data Bank 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, groupd, and individual B-factor refinement. A constant bulk solvent model was applied throughout the procedure. 5% of the data were used to calculate

### Table 1

| Data set | GH1_26 |
|----------|--------|
| c(Ca⁺) (mM) | 50     |

| Data collection | X-ray source | ESRF (BM14) |
|-----------------|--------------|-------------|
| Wavelength (Å)  | 0.978        |             |
| Space group     | P321         |             |
| Unit cell dimensions (Å) | 132.7, 132.7, 61.3 |
| Matthews coefficient (Å³/Da)/solvent content (%) | 4.3/72 |
| Resolution (Å)  | 2.5          |             |
| No. of measurements | 120,728     |
| No. of independent reflections | 21,804 |
| Multiplicity (%) | 5.5 (5.5)   |
| Completeness (%) | 100 (100)   |

| Refinement | No. of reflections in working set | 20,672 (1522) |
|------------|----------------------------------|---------------|
|            | No. of reflections in test set   | 1,116 (85)    |
|            | No. of non-H protein atoms       | 2,832         |
|            | No. of water molecules           | 261           |
|            | No. of calcium ions              | 3             |
|            | No. of phosphate ions            | 5             |
|            | Average B-factor (Å²)            | 25.3          |
|            | Ramanchandram plot (%)<sup>a</sup> | 90.5/8.8/0.7/0 |
|            | r.m.s.d. B-factor for bonded atoms (Å²) | 1.231 |
|            | r.m.s.d. bond lengths (Å)        | 0.011         |
|            | r.m.s.d. bond angles (°)         | 1.641         |
| R<sup>b</sup> | 0.193 (0.267)                   |
| R<sub>free</sub> | 0.252 (0.319)                  |

<sup>a</sup> Residues in the most favored/additionally allowed/generously allowed/disallowed region.

<sup>b</sup> R = Σ||Fo| − |Fc||/Σ|Fo|, where F₀ and Fc are the observed and calculated structure factors, respectively.

<sup>c</sup> R<sub>free</sub> is defined in Ref. 56.
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 1. Coordinates and structure factors of GH1_26 have been deposited with the Protein Data Bank under accession number 3brx.

Preparation of Phospholipid Vesicles—Small unilamellar vesicles (SUVs)2 with diameters ranging from 15 to 50 nm were prepared by dispersion of multilamellar vesicles using sonication. The preparation procedure follows that of Pagano and Weinstein (41) with some modifications. 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 multilamellar vesicle solution. Subsequently, bath sonication was performed to disperse the multilamellar vesicles 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 color.

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); the titration syringe contained a solution of 50 mM CaCl$_2$ in the absence of vesicles 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 at 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 possible only by exchanging the major precipitant from sulfate to phosphate salts and rather high calcium concentrations. Although soaking the protein crystals with high calcium concentrations cracked the crystals and rendered them unusable, co-crystallization at high levels of calcium required precipitants other than sulfate salts. At low calcium concentrations, the crystal shape is triangular prism-like, and the size of crystals decrease with increasing calcium concentrations (see Fig. 1). Crystals of annexin Gh1 obtained under 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 P3$_1$21 to P3$_21$, 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 Fig. 2 and Table 2). In the IAB loop, a calcium ion (CA1) is coordinated with pentagonal bipyramidal geometry. Three carbonyl oxygen atoms (Phe-29, Gly-31, Gly-33), the bidentate carboxylate of Glu-73, 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 Trp-104 from the IAB loop of a symmetry-related molecule generated by the 3-fold axis. The involvement of Trp-104 in calcium coordination distorts the backbone geometry of Trp-104 ($\phi = 70^\circ$, $\psi = -90^\circ$) and Thr-

2 The abbreviations used are: SUV, small unilamellar vesicle; DMPC, dimyristoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; ITC, isothermal titration; r.m.s.d., root-mean-square deviation.
105 \( (\phi = -170^\circ, \psi = 40^\circ) \) from the standard parameters \((44)\), thus making these residues the outliers in the Ramachandran plot. Another calcium ion \((\text{CA2})\) is bound to the IVAB loop, which has moved closer to the IVDE loop when compared with the crystal structure of the apo-protein. Coordination for CA2 is provided by three backbone carbonyl oxygen atoms \((\text{Ile-259, Arg-261, Gly-263})\) and the bidentate carboxylate of Asp-303, thus constituting a trigonal-bipyramidal geometry. This coordination geometry of a type II binding site has been observed earlier in other annexin crystal structures \((\text{Protein Data Bank accession numbers 1avh, 1aii, 1xjl})\) \((45–47)\). A third calcium ion \((\text{CA3})\) is bound in the IVDE loop and coordinated by two carbonyl oxygen atoms \((\text{Val-301, Thr-304})\), the bidentate carboxylate of Glu-309, two water molecules, and a phosphate ion acting as a monodentate ligand. CA3 thus occupies a type III binding site.

Superposition of the current structure with other structures of apo-plant annexins shows the usual conformational divergence with r.m.s.d. of 2.4–3.0 Å. Compared with the structure of apo-annexin Gh1 \((r.m.s.d. = 1.3 \text{ Å})\), the only significant conformational differences occur in loops IAB, IDE, IIAB, IVAB, and IVDE \((\text{see Fig. 3})\). Loop IVAB moves closer toward loop IVDE, with Thr-264 relocating by 9 Å. The second-most significant movement is observed in loop IIAB, where Trp-104 moves about 5 Å and the guanidinyl group of Arg-103 by about 7 Å. The semi-sheltered position of Trp-104 in the apo-structure, where Arg-103 shields the tryptophan residue, has changed to fully expose Trp-104 and Arg-103.

### FIGURE 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 2\(F_o - F_c\) electron density is contoured at 1.7\(\sigma\). This figure was prepared using PyMOL \((53)\).

### TABLE 2
Calcium coordination geometry

| Binding site | Type | Coordination | Backbone carbonyls | Bidentate side chains | Solvent |
|--------------|------|--------------|--------------------|----------------------|---------|
| CA1 IAB     | II   | 7            | Phe-29: 2.37; Gly-31: 2.58; Gly-33: 2.53; Trp-104*: 2.41 | Glu-73: 3.04 (OE1), 2.71 (OE2) | HOH-3: 2.93 |
| CA2 IVAB    | II   | 5            | Ile-259: 2.56; Arg-261: 2.12; Gly-263: 2.12 | Asp-303: 2.44 (OD1), 2.78 (OD2) |         |
| CA3 IVDE    | III  | 7            | Val-301: 2.15; Thr-304: 2.67 | Glu-309: 2.91 (OE1), 3.00 (OE2) | HOH-96: 2.96; HOH-134: 2.38; PO_{4}^{3-}: 2.50 (O-4) |
The carbonyl oxygen of Trp-104 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 rearrangement is observed upon calcium binding. Glu-73 moves downward, and the backbone of Trp-32 moves toward the IDE loop, each by 2 Å. Occupation of the type III binding site in the IVDE loop leads to a slight repositioning of the backbone carbonyl groups of residues 301 and 304 by 2.5 and 1.5 Å, respectively.

Neither of the two exposed tryptophan residues is harbored in a predominantly hydrophobic pocket. The conjugated π-electron system of Trp-32 is in stacking conformation with Arg-80 from the top part of the IE helix of a neighboring molecule, indicating hydrogen bonding. The side chain of Trp-104 faces a pocket presented by Lys-66, Ala-69, Lys-72, and Glu-73 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 (Fig. 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 no association of calcium ions with cotton annexin Gh1 in solution. A similar phenomenon is well known for several animal annexins, which show only a 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 (Fig. 4). The fit parameters reveal a stoichiometry of 4.35 ± 0.154 mol of calcium/1 mol of annexin Gh1. The apparent dissociation constant for the ternary complex annexin Gh1-Ca²⁺-membrane is $k_d = 11 \mu M$.

This results in a $c$-value for 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 the secondary structure upon unfolding of the protein (see Fig. 5 and summary of results in Table 3). Surprisingly, two transitions were observed for the plant annexins tested in this study.

The first transition, which occurred at about 28 °C, was not dependent on the presence of calcium. The reason for this additional transition is not entirely clear, as the elongation of the N-terminal domain by the poly-His fusion is not the cause. However, because the N-terminal domain is anchored to the C-terminal core in all crystal structures of plant annexins available to date, this phenomenon might be the reason for the additional transition (see “Discussion”).

The second transition corresponded to the main unfolding of the protein secondary structure and happened, in the absence of calcium, at $T_{m} = 63$ and 58 °C for the native and the His₆-tagged bell pepper annexin. Both proteins were thus more stable than annexin A5. The His₆ fusion construct of cotton annexin Gh1 was slightly less stable than annexin A5 ($\Delta T_{m} = -2$ K). In the presence of 10 mM calcium, all plant annexins tested were destabilized, as indicated by the lower transition temperatures for the secondary structure unfolding. The temperature difference was $-8$ and $-5$ K for wild-type and fusion bell pepper annexin, respectively; for cotton annexin Gh1, the temperature difference was $-10$ K. This phenomenon is in sharp contrast to the situation found with mammalian annexins such as AnxA5 (50), which are usually stabilized 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 predicted previously (30). Analysis of the conformations in both the apo- and calcium-bound crystal structure indicated 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 with 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 Trp-35, is striking (see Fig. 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 hypothesize 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 Trp-104, 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 emphasizes 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 were observed mainly with side chain conformations. No major rearrangements were 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), bringing the AB loop in closer position to the DE loop and 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, although domain II does not harbor a functional calcium binding site, IIAB 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 was observed in the IIAB loop. Although Arg-103 and Trp-104 adopted semi-sheltered positions in the apo-structure, both residues were now fully exposed and were probably directly interacting with phospholipid molecules in the membrane-bound state, which is in agreement with our previous results (31). The unfavorable exposure of Trp-104 to solvent in the absence of a membrane was compensated by the crystal packing. In the present structure, Trp-104 contributed to the coordination of a calcium ion in the IAB loop of a symmetry-related molecule, which energetically stabilized the exposed conformation.

**Calcium Binding and Implications for the Membrane-bound State**—Calcium titration of annexin Gh1 in solution using isothermal titration calorimetry revealed 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, as no calcium-correlated change of the signal was 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 yielded a molar ratio of 4 calcium ions/protein molecule in the membrane-bound state.

Although only three calcium ions were located in the present crystal structure, analysis of the IDE loop region of the calcium-bound crystal structure of annexin Gh1 showed that a water molecule occupies the putative type III binding site of the first repeat. The backbone carbonyl oxygen atoms of Asp-71 (2.56 Å) and Leu-74 (2.58 Å), as well as the bidentate carboxylate group of Glu-79 (OE1: 3.44 Å, OE2: 2.75 Å) adopt a conformation that would enable coordination of a calcium ion instead of water molecule HOH-115. Other possible contributing ligands include the carboxyl group of Asp-71 as monodentate ligand (OD2: 3.27 Å) and another water molecule, HOH-123 (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 hypothesize that the canonical membrane-bound species of cotton annexin Gh1 utilizes the type II and type III calcium binding sites in repeats I and IV for the

FIGURE 6. The conformational switch of the IAB loop in plant annexins. Calcium-bound cotton annexin Gh1 is shown in red, apo-annexin Gh1 in brown, and the two conformations of bell pepper annexin 24(Ca32) in green. This figure was prepared with MolScript (54) and rendered with Raster3d (55).

TABLE 3

| Protein          | T_{1/2}   | D(T_{1/2}) | Without Ca^{2+} | 10 mM Ca^{2+} |
|------------------|-----------|------------|-----------------|---------------|
|                  | °C        | °C         |                 |               |
| 1st transition   |           |            |                 |               |
| Annexin 24(Ca32)| 30        | 30         | ±0              |               |
| His_{6}-annexin 24(Ca32) | 26 | 25 | −1 |
| His_{4}-annexin Gh1 | 26 | 24 | −2 |
| Annexin A5*     |           |            |                 |               |
| 2nd transition   |           |            |                 |               |
| Annexin 24(Ca32)| 63        | 55         | −8              |               |
| His_{6}-annexin 24(Ca32) | 58 | 53 | −5 |
| His_{4}-annexin Gh1 | 48 | 38 | −10 |
| Annexin A5*     | 52        | 59         | +7              |               |

* The values for human annexin A5 are given for comparison. Data are from Ref. 50.
calcium-dependent binding mode. Plant annexins thus 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 Trp-32 and Trp-104 with the phospholipid head group or glycerol backbone of the lipid membrane. The conformations found for other surface residues indicate that Arg-103, Lys-187, Lys-230, and Arg-262 will also engage in direct contact with the membrane.

**Protein Fold Stability**—Thermal denaturation experiments of the three plant annexin constructs in this study revealed 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, because 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-3/Thr-3 and His-40 (annexin Gh1 numbering). Analysis of the known plant annexin structures showed that His-40 is hydrogen-bonded to the backbone carbonyl of residue 5 and the side chain hydroxyl group of Ser-3 or Thr-3 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 was 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 from the situation found in annexins in other kingdoms, where the N-terminal domain of individual proteins is unique in their primary, secondary, and tertiary structures. Further experiments are needed to reveal the functional significance of this feature.

In contrast to mammalian annexins, all plant annexins tested in this study were 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 destabilizing effect of calcium might thus be explained by a stabilization 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 destabilized 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 His-tagged annexin 24(Ca32), respectively.

**Conclusion**—The property of the calcium-dependent membrane binding of plant annexins is well established. Recently, we have shown that these proteins possess a second membrane binding mode that 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 located 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, because we determined a stoichiometry of 4 calcium ions/protein molecule in the membrane-bound state of annexin Gh1.

The high similarity of cotton annexin Gh1 to 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 to determine the workings of plant annexins in cellular processes.

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