Title
A calcium-driven conformational switch of the n-terminal and core domains of annexin A1

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Annexin 1 Crystal Structure:

Interaction of Annexins with Membranes

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Abstract

Annexins are structurally divided into a highly conserved core domain and a variable N-terminal domain. The core domain mediates the calcium-dependent phospholipid binding of annexins, whereas the N-terminal domain, which is unique in sequence and length for each member of this protein family, is responsible for the specificity among the different members. Annexin 1 has been shown to possess membrane aggregation and fusion activity in the presence of calcium in vitro. Due to the high sequence homology of the core domain among different annexins, the property of membrane aggregation has been attributed to the N-terminal domain. For instance, a chimera protein comprising the core of annexin 5, which by itself does not exhibit membrane aggregation properties, and the N-terminal domain of annexin 1 is able to induce membrane aggregation. Numerous three-dimensional structures of annexins have been solved using x-ray crystallography, however, none reveal the tertiary structure of an N-terminal domain or its interaction with the core domain. We have solved the x-ray structure of full-length annexin 1 with an N-terminal domain comprising 42 amino acids in the absence of calcium ions at 1.8 Å resolution. Residues 2-26 of the N-terminal domain exhibit a mainly α-helical conformation with a kink at residue 17. Helix NA (residues 2 to 16) inserts into repeat III of the core domain thereby replacing the old helix D. Helix D on the other hand unfolds into an extended loop that forms a flap over the top of helix NA of the N-terminal domain. As a result, the type II calcium-binding site located in core repeat III is destroyed because the "capping residue" for calcium ion coordination is not in the proper conformation/location any more. Also presented in this article is the structure of full-length annexin 1 in the presence of 1 mM $CaCl_2$. The structure of full-length annexin 1 in the absence of calcium ions is thought to represent the "inactive" form of the protein. We provide a model for the annexin 1-induced membrane aggregation and discuss it in light of the literature published to date.

Introduction

Over the past 20 years, annexins have been described as calcium- and phospholipid-binding proteins. Biochemical and cell biological experiments have been conducted in order to elucidate the properties of different annexins in vitro to further define their biological role. Annexins share a high sequence homology among their core domains, and the core domain is responsible for calcium-dependent lipid binding. The unique properties of each annexin depend on the N-terminal domain, which is different in sequence and length for each member of this protein family.[1-4]

Besides biochemical work, x-ray crystallography has been used in order to reveal the structural requirements for calcium- and membrane binding of several different annexins. In 1990 and 1992, Huber and co-workers published the first annexin structure, i.e., that of annexin 5 (PDB code: 1AVH & 1AVR).[5,6] The overall shape of the protein can be described as a curved disk with the calcium ions located on the convex site of the disk. This disk is composed of four

Annexins, edited by Joanna Bandorowicz-Pikula.

repeats, which contain five α-helices (named A through E) each, while the loops that connect α-helices A/B and D/E harbor the calcium-binding sites. Two new calcium-binding motifs have been determined in annexins—type II and type III binding sites. They are different from the type I, or EF-hand, calcium-binding sites, with respect to the coordination of the calcium ion. In the type II binding-site the calcium ion is coordinated by three backbone carbonyl oxygen atoms located in the AB loop, the side chain of an acidic amino acid that resides approximately 40 residues downstream in sequence from the AB loop (Asp or Glu) and two water molecules (pentagonal bipyramidal coordination). Type III binding sites provide a different coordination: the calcium ion is coordinated by two backbone carbonyl oxygen atoms, one side chain of an acidic residue and three water molecules.

The prevailing view that calcium-dependent membrane binding occurs via the type II sites on the convex surface of the protein was supported by x-ray structures of annexin 5 in the presence of calcium ions and lipid analogs (PDB-code: 1A8A and 1A8B).[7] At this point, the calcium-dependent phospholipid-binding of annexins could be explained, but the mechanism of membrane aggregation and fusion still remained obscure. Annexins I and II have been reported to exhibit membrane aggregation properties. Although their respective core domains are very similar to annexin 5, these two annexin species contain N-terminal domains with more than 30 amino acid residues (annexin 5 has an N-terminal tail of 16 residues). The N-terminal domain of annexin 1 contains 42, and that of annexin 2 contains 32 amino acid residues.

In 1993, the x-ray structure of an annexin 1 derivative lacking the first 32 amino acid residues was published by Huber and co-workers (PDB-code: 1AIN).[8] The structure revealed a typical annexin core domain, very similar to the one of annexin 5.[6] Calcium ions were bound in the AB-loops of each of the four repeats, plus two extra ones in the DE-loops of repeat I and repeat IV. Due to the unanticipated proteolytic removal of the first 32 amino acid residues, no information was available on the structure of the N-terminal domain itself or its interaction with the core domain. Furthermore, no explanation was apparent as to why annexin 1 is capable of membrane aggregation and annexin 5 is not. Obviously, the N-terminal domain of annexin 1 plays an important role in the membrane binding activities described in these biochemical and structural experiments.

Annexin 1, the protein of interest in our research during the last six years, has been shown to aggregate chromaffin granules and artificial membranes in the presence of calcium ions.[9-13] Membrane fusion activity has also been reported.[11,13] A multitude of biochemical experiments have been conducted during the last ten years in order to understand the mechanism of the annexin 1 induced membrane aggregation. Unfortunately the results are quite contradictory:

Ernst and co-workers tested an annexin 1/5 chimera in which they fused the first repeat of annexin 1 (plus eight amino acid residues of the N-terminal domain) to repeats II to IV of annexin 5. Annexin 5 has not been shown to promote membrane aggregation, whereas this chimera did.[14] Reutelingsperger's group chose a different approach: they designed a chimera comprising the first 45 residues of annexin 1 fused to the core domain of annexin 5 (residues 19-320). This chimera was also able to promote membrane aggregation, leading to the hypothesis that the N-terminal domain of annexin 1 alone was responsible for membrane aggegation.[15,16] However, in 1994 Creutz and co-workers studied the aggregation properties of proteolytically cleaved isoforms of annexin 1, and interestingly they could show that a truncation at Lys26 resulted in a protein (Δ1-26 annexin 1) that mediated half-maximal membrane aggregation in the presence of 32 μM Ca^{2+}. Full-length annexin 1 and Δ1-29 annexin 1 required 63 μM Ca^{2+} whereas the Δ1-12 annexin 1 derivative required 0.1 mM Ca^{2+} to induce comparable aggregation.[12] Cho and co-workers published similar results in the late 1990s. This group worked with truncated annexin 1 derivatives as well as mutant proteins. From theses studies it became evident that the residues Lys26 and Lys29 themselves play a role in

membrane aggregation.[17] The N-terminal domain is obviously very important for the annexin 1-induced membrane aggregation, however, from all the above mentioned studies no mechanism could be derived.

Various research groups have described another interesting feature of annexin 1: after calcium-dependent binding of annexin 1 to lipids, a secondary membrane-binding site becomes accessible. Interestingly, this secondary binding site exhibits neither calcium dependency nor lipid specificity.[9,13,18-20] These data suggest that the N-terminal domain of annexin 1 alone might provide a second membrane-binding site. This hypothesis is supported by a publication from Lee and co-workers in which they present data that a peptide comprising residues 1-26 of annexin 1 alone is able to form an α-helix in 50% trifluorethanol (TFE)/water and 10 mM sodium dodecyl sulfate (SDS), suggesting membrane binding activity.[21]

We are interested in the annexin 1-induced membrane aggregation and even fusion of membranes in vitro and in vivo. As described above a lot of biochemical data is available on the aggregation and fusion properties of annexin 1, however, the structural requirements for this interaction have not been examined in detail.

The Three-Dimensional Structure of Full-Length Annexin 1 in the Absence of Calcium Ions

Full-length recombinant porcine annexin 1 was expressed and purified according to Rosengarth et al (1999).[22] Crystals were grown in 2.2 M ammonium sulfate, 0.1 M Tris-HCl, pH 8.5 at 4°C.[23] Native crystals diffracted to better than 1.8 Å at the Advanced Light Source (ALS) in Berkeley, beamline 5.0.2 and the structure was solved using a combination of the Molecular Replacement (MR) and the Multiple Isomorphous Replacement (MIR) technique.[24]

After mass spectrometry showed that the protein in the crystals was the full-length protein,[23] the question arose: how does the N-terminal domain fold? Secondary structure prediction using the program SOPMA for the N-terminal domain suggested a highly α-helical secondary structure for residues 1–26 including a kink at positions 16 and 17. Residues 27–42 were predicted to be in a random coil conformation.[25] In agreement with this prediction is the x-ray structure of an N-terminal peptide of annexin 1 comprising residues 2-15 in complex with the S100A11 protein in which the peptide adopts an α-helical conformation (PDB-code: 1QLS).[26] Our structure of full-length annexin 1 is in excellent agreement with the predictions and peptide conformations—the first 26 amino acid residues adopt an α-helical conformation with a kink at residue 17. However, the N-terminal domain is not simply located at the concave side of the molecule only as one might have expected from the structure of annexin 1 lacking the first 32 amino acid residues.

Shown in Figure 1 is a comparison of the x-ray structures of the annexin 1 derivative lacking the first 32 amino acid residues from N-terminus (PDB-code: 1AIN) and the full-length protein (PDB-code: 1HM6). The overall structure of the full-length protein still resembles a typical annexin core domain, however no calcium ions are bound. Shown in yellow is the N-terminal domain of annexin 1, including the two α-helices (named NA and NB) with a kink at residue 17. As mentioned before, the N-terminal helices are not just an extension of the N-terminal tail shown in the annexin 1 structure without the complete N-terminal domain. Surprisingly, the helices of the N-terminal domain interact with repeat III of the core domain by replacing the D-helix of this repeat. As a result, the former D-helix unfolds into an extended loop that is located on top of the NA helix, like a flap.

Figure 2 illustrates the conformational change observed in the full-length structure of annexin 1 in comparison to the derivative structure in more detail. Inspecting this region in stereo makes it even easier to see the dramatic conformational change in repeat III. The first four to five amino acid residues of the amphipathic helix NA insert into the same place that is occupied by part of the D-helix in the truncated annexin 1 structure. Helix C in the full-length structure is shorter as in the truncated structure, therefore making the extended loop formed

by the former D-helix even longer. Also very noticeable is the fact that the extended loop of the former core domain D helix now folds over the NA helix of the N-terminal domain like a flap, burying parts of the hydrophilic side of the amphipathic helix (see Fig. 3). Further examination of the electron density for residues 2-8 reveals favorable packing of the hydrophobic residues on the other side of this amphipathic helix (Met3, Val4, Phe7) into the hydrophobic pocket formed by residues Phe221, Leu225, Phe237 and Val268 of repeat III.

The Crystallographic Annexin 1-Dimer and Consequences for Calcium Binding

Full-length annexin 1 crystallizes as a dimer in the absence of calcium ions. It is very likely that the observed dimer is a crystallographic rather then a biological one. The dimer contacts occur via the Lys250 residues, whose ε-amino groups are coordinated by three backbone carbonyl oxygen atoms in the AB-loop of repeat II in the respective opposing monomer, thereby replacing the calcium ion observed in the truncated annexin 1 derivative.[8] Therefore, the lysine ε-amino group replaces the calcium ion coordinated in repeat II that was observed in the structure of annexin 1 lacking the first 32 amino acid residues (see Fig. 4). The replacement of calcium ions by lysine residues has also been observed in the case of the annexin 12 hexamer and the annexin 12 mutant protein E105K (also a hexamer).[27,28] If the observed dimer were of biological significance, the protein could not bind to the membrane via the calcium ions anymore. It has been shown that especially the calcium-binding site in repeat II is of great importance for membrane binding.[29] Additionally, the type II binding-site in repeat III is destroyed as well, because the helix D that is now unfolded into an unstructured loop no longer supplies the capping residue (Glu255) for calcium coordination. Another reason to believe that the dimer we observed in the crystal structure is not a biological one is that we could not detect dimers in solution using dynamic light scattering (data not shown).

Model for the Annexin 1-Induced Aggregation of Membranes: Monomeric Annexin 1 Displaying Two Membrane-Binding Sites

The cartoon shown in Figure 5 illustrates a possible mechanism of the annexin 1-induced aggregation of membranes. This model is supported by our x-ray structure of full-length annexin 1 and by results published by other groups over the last ten years.[13,18,20,21,24] In this model, the structure of full-length annexin 1 in the absence of calcium ions reflects the starting point of the reaction cascade that eventually causes membrane aggregation. This reaction cascade would start with calcium-dependent binding of one annexin 1 monomer to a membrane containing negatively charged phospholipids. During this binding event, the N-terminal helix NA would be expelled from the core of the protein, because helix D of repeat III is required to refold for calcium-dependent lipid binding (step 1). Once the N-terminal domain is not shielded by the annexin core any more, its amphipathic helix NA could interact with another membrane (step 2). This secondary membrane binding is not calcium-dependent and non-specific with respect to the lipids in the membrane.[13,20] This scheme proposes that the secondary lipid-binding site in annexin 1 is provided by the N-terminal helix NA. Residues 2-26 of annexin 1 have been shown by NMR experiments to adopt a helical conformation in 50%TFE/water and 10 mM SDS, suggesting that this peptide interacts with membranes.[21]

The mechanism depicted in Figure 5 is one possibility for annexin 1 to be part of the membrane aggregation and fusion machinery. Other possibilities include the dimerization of two annexin 1 molecules via their N-terminal amphipathic helices (hydrophobic interaction!) or the formation of a heterotetramer composed of two annexin 1 monomers and one S100A11 dimer.[30,31] It has been observed by cryo-electron microscopy that annexin 1 bridges dioleoylphosphatidylglycerol (DOPG)-dioleoylphosphatidylcholine (DOPC) pairs of bilayers

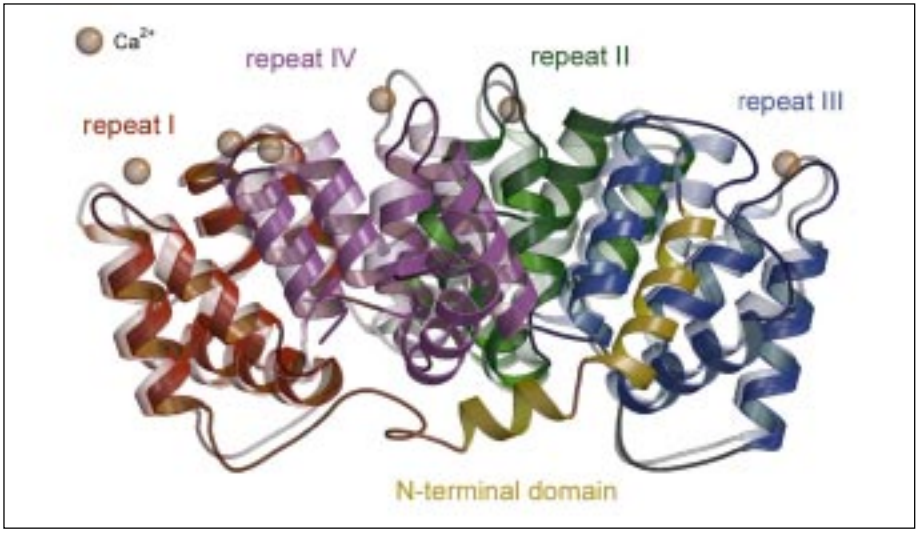

Figure 1. Overlay of the ribbon diagrams of one monomer of recombinant porcine annexin 1 comprising protein core and the N-terminal domain (solid rendering, PDB-code: 1HM6)[24] with human annexin 1 lacking the first 32 amino acids shown (slightly shifted transparent rendering, Δ1-32 anx1; PDB-code 1AIN).[8] Repeat I is presented in red, repeat II in green, repeat III in blue, repeat IV in purple and the N-terminal domain in yellow. The yellow N-terminal helix in the full-length structure is replacing the two-turn blue helix in the core of the Δ1-32 annexin 1. Bound calcium ions in Δ1-32 annexin 1 are illustrated as yellow spheres.

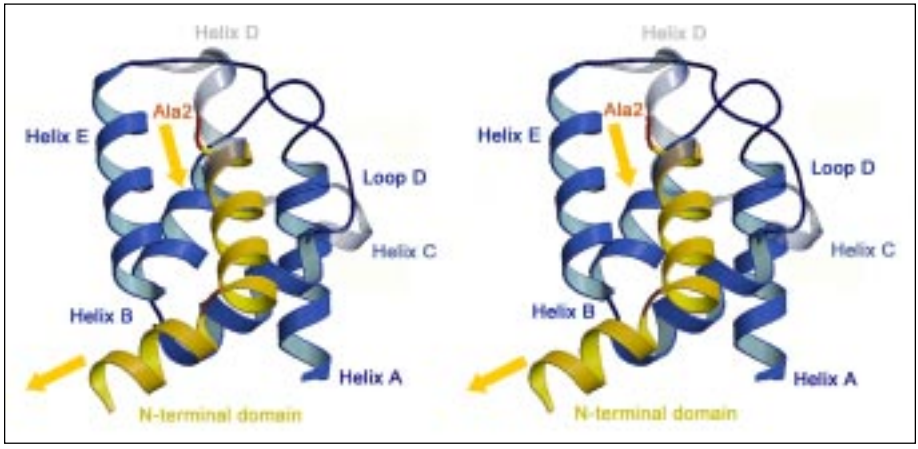

Figure 2. Stereo overlay of the three-dimensional structures of repeat III of full-length annexin 1 comprising helices A, B, C and E (blue) plus residues 2 to 26 of the N-terminal domain (yellow) with repeat III of Δ1-32 annexin 1 (gray). Note that helix D of Δ1-32 annexin 1 (gray) unwinds into an extended loop (loop D, dark blue) in the full-length structure, which forms a flap over the N-terminal helix. The yellow arrows indicate the direction of the N-terminal α-helices, which are connected to repeat I of the core domain via an extended linker.

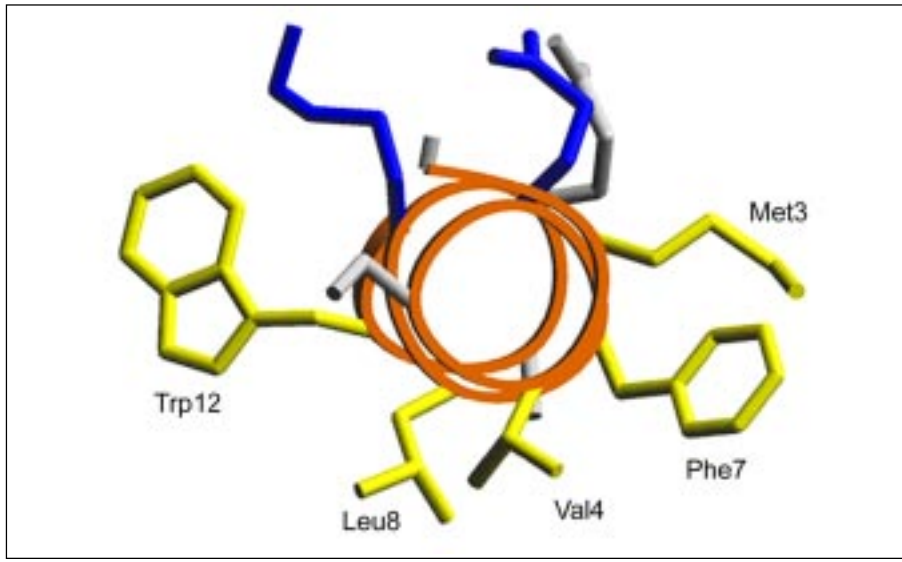

Figure 3. Helical wheel representation of the amphipathic helix NA (residues 2-12): view from the N-terminus down the helix axis shows that the bottom face is lined with hydrophobic side chains (Met3, Val4, Phe7, Leu8, Trp12) (yellow) while the top face is lined with hydrophilic residues (Glu6, Lys9) (blue).

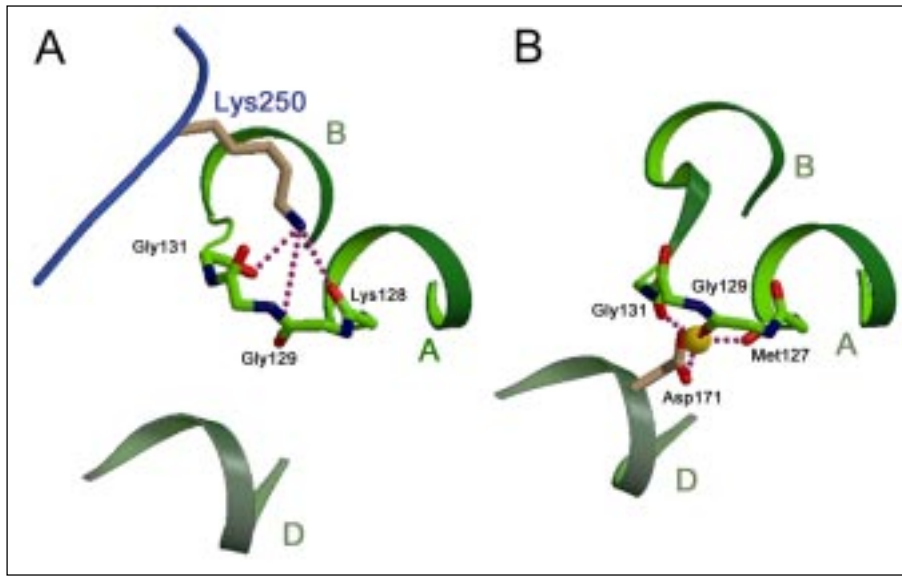

Figure 4. Calcium mimicry in the AB loop in repeat II of annexin 1. (A) The coordination of the ε-amino group of Lys250 in the type II binding site of repeat II by backbone carbonyl oxygen atoms of Lys128, Gly129 and Gly131 residues in the same loop in the absence of calcium in the full-length annexin 1 structure. (B) The coordination of the calcium ion (yellow) in the type II binding site of repeat II as reported in the Δ1-32 annexin 1 structure. Backbone carbonyl oxygen atoms of Met127, Gly129 and Gly131 and the carboxylate OD1 and OD2 of Asp171 are coordinating the calcium ion (two water molecules complete the coordination sphere but are not shown).

in the presence of calcium ions in two different ways: one could be the juxtaposition via an annexin 1 dimer (corresponding to observed layer of 160 Å thickness between the membranes), the other via an annexin 1 monomer (corresponding to observed layer of 125 Å thickness).[32]

The Structure of Full-Length Annexin 1 in the Presence of Calcium Ions

In the proposed model of annexin 1-induced membrane aggregation via one annexin 1 monomer harboring two distinct membrane interaction sites, the prerequisite for secondary calcium-independent membrane binding is a primary calcium-dependent binding event. We believe that upon calcium and/or membrane binding the N-terminal helix NA of annexin 1 is expelled from the core. One way to investigate this hypothesis is to determine the x-ray structure of full-length annexin 1 in the presence of calcium ions. Unfortunately, this task has not been easy to accomplish: so far, in spite of numerous screening trials, no crystals could be grown in the presence of Ca^{2+}. This observation fits into the picture in that the NA helix is probably expelled from the core and may not be in a fixed three-dimensional conformation. Consequently, crystallization contacts might be difficult to form.

Instead, we were able to collect diffraction data on crystals of full-length annexin 1 that were soaked in a calcium solution. We sequentially soaked the crystals in mother liquor containing 0.1 mM, 0.5 mM and 1 mM calcium chloride for one hour at 4°C. Any attempts to increase the calcium concentration or the incubation time resulted in cracking of the crystals, probably an indication of a large conformational change upon calcium binding. This conformational change could include the disruption of the crystallographic dimer and/or the release of the NA helix from the core. These step-wise soaked crystals diffracted to around 2.3 Å (for data and refinement statistics see Table 1).

Overall, we could not detect a major conformational change upon Ca^{2+} binding at 1 mM $CaCl_2$, i.e., not many structural differences could be found between the calcium-free and the calcium-bound form of the protein. The root mean square deviation between monomer B of the calcium-free vs. the calcium-bound form is only 0.95 Å. However, we were able to detect three bound calcium ions per dimer: two are bound in the AB-loop of repeat I of each monomer, and these positions coincide with calcium sites in the truncated annexin 1 structure. Interestingly, the third calcium ion, we were able to localize, is bound in the AB-loop of repeat II in monomer B. The overlay of this calcium site with Lys250 binding in the calcium-free form of full-length annexin 1 in the AB-loop of repeat II (monomer B) is shown in Figure 6A. The coordination of the ε-amino group of Lys250 by backbone carbonyl oxygen atoms in AB-loop of repeat II of the other monomer in the calcium-free form takes place on the other site of the loop in comparison to the calcium coordination of the soaked form. Interestingly, the data collected on the calcium-soaked crystals seem to reflect a kinetic intermediate between calcium-free and calcium-bound form because we observe calcium as well as lysine ε-amino-binding. On one face of the AB-loop the calcium ion is bound, whereas on the other side we still observe the coordination of the ε-amino group of Lys250 of the other monomer. However, the arrangement of the residues/atoms is slightly different (see Fig. 6B): the ε-amino group of Lys250 is moved by 0.75 Å.

Although the crystallographic dimer seems to dissociate upon calcium binding, the N-terminal helix NA is still in close contact with the core in the presence of 1 mM $CaCl_2$. In solution, it has been shown that one annexin 1 monomer binds two calcium ions in the presence of 1 mM $CaCl_2$.[33] According to the x-ray structures, these two calcium ions are probably bound in the AB-loops of repeat I and II. The third "high-affinity" calcium-binding site is located in the AB-loop of repeat III. However, this binding site is not accessible for ion binding because of the N-terminal NA helix interacting with repeat III, thereby replacing the D-helix,

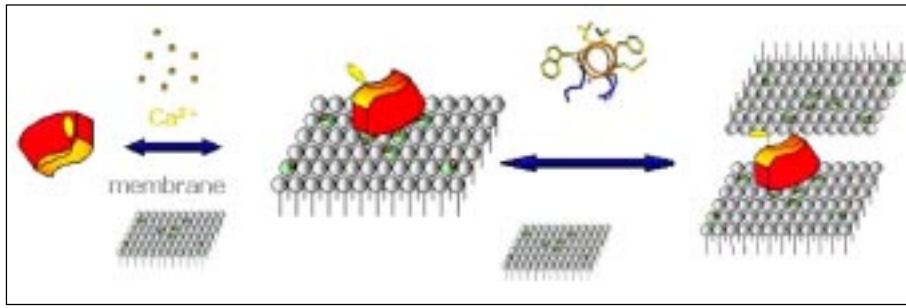

Figure 5. Model of annexin 1-monomer-induced membrane aggregation. The inactive form of the protein, in the absence of calcium ions, is represented as the molecule with the N-terminal domain (yellow) buried in the core domain on the left. Upon calcium and membrane binding (membrane from below), the extended loop of repeat III folds into the D-helix, resulting in proper type II site conformation for calcium binding. As a result, the N-terminal domain is expelled from the hydrophobic pocket of repeat III of the core domain. Now, the N-terminal domain is exposed to the bulk solvent, allowing its amphipathic α-helix (residues 2-12) to interact with a second membrane (monolayer pictured above). In this membrane bridging mode, one annexin 1 monomer would display two different membrane interaction sites: a calcium-mediated one that interacts with anionic phospholipids on its convex side (bottom) and a secondary one that interacts non-specifically with bilayers through the amphipathic helix on the concave side (top).

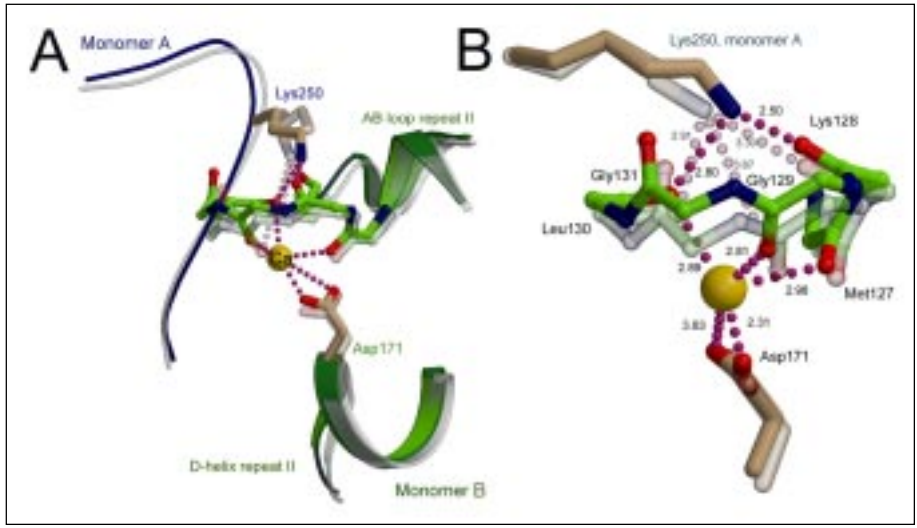

Figure 6. Comparison of the calcium-binding site in the AB loop of repeat II of full-length annexin 1 (monomer B) in the absence (transparent rendering) and presence (solid rendering) of calcium. (A) Overlay of the calcium- (solid) and Lys250-coordination (transparent) in the AB-loop of full-length annexin 1. On one face of the AB-loop the calcium ion is bound, whereas on the other side we still observe the coordination of the ε-amino group of Lys250 of the other monomer. (B) A closer look at the calcium binding site reveals that the arrangement of the residues/atoms is slightly different: the ε-amino group of Lys250 has moved by 0.75 Å and therefore the coordination of the ε-amino group occurs via backbone carbonyl oxygen atoms of the Lys128 and Gly131 residues only. The coordination of the calcium ion (yellow) in the type II binding site of repeat II is as reported in the Δ1-32 annexin 1 structure (PDB-code: 1AIN).[8] Backbone carbonyl oxygen atoms of Met127, Gly129 and Gly131 and the OD1 and OD2 of Asp171 are coordinating the calcium ion (two water molecules complete the coordination sphere but are not shown in here).

Table 1. Data collection and refinement statistics

Data Collection Statistics	
Space group	$P2_12_12_1$
Unit cell dimensions	a = 63.194 Å, b = 95.941 Å, c = 127.804 Å
Resolution range (Å)	99.0 - 2.3
Number of observations	494,391
Unique structure factors	53,997
R_{merge}* (%) all/2.36 – 2.30 Å	0.15 / 0.535
I / σ_I all/2.36 – 2.30 Å	20 / 2.9
Completeness all/2.36 – 2.30 Å	90 / 97.9
Mosaicity (°)	0.471
Refinement Statistics	
Refinement resolution range (Å)	50.0 - 2.3
Reflections used for refinement (working set)	32,269
R-factor (%), no sigma cutoff	23.99
R_{free} (%), no sigma cutoff	28.41
R.m.s. standard deviations	
Bond lengths (Å)	1.334
Bond angles (°)	2.18
Average B value for Cα atoms, monomer A (Å^2)	43.0
Average B value for Cα atoms, monomer B (Å^2)	43.4
Average B value for water molecules (Å^2)	42.5
Protein atoms (two annexin 1 molecules)	5,394
Water molecules	267
Sulfate ions/atoms	11

*$R_{merge}(I) = \Sigma_{hkl}\Sigma_i \mid I_{hkl,i} - \langle I_{hkl}\rangle \mid / \Sigma_{hkl}\Sigma_i \mid I_{hkl,i} \mid$, where $\langle I_{hkl}\rangle$ is the average intensity of the multiple $I_{hkl,i}$ observations for symmetry-related reflections.

which is necessary for calcium binding. The affinity of the type II binding site in repeat IV is unexpectedly low in comparison to that of the type III binding site in the AB-loop of repeat I because no calcium ions could be detected in this site.

From calcium binding studies in solution and proteolysis experiments one could infer that the N-terminal helix is expelled either at Ca^{2+} concentrations as high as 100 mM or upon membrane binding in the presence of calcium ions.[19,33] It has been shown that membrane-bound annexin 1 is approximately 40 times more sensitive to trypsin (which cleaves at position Lys26) and cathepsin D (which cleaves at residue 12) than free annexin 1.[19] Therefore, the N-terminal domain of annexin 1 is more accessible as a result of calcium-dependent membrane binding than in the calcium-free from. These results are in good agreement with our hypothesis that the helix NA is only accessible after calcium and/or membrane binding. Additionally, it has been suggested that the core domain is involved in a conformational change that leads to the exposure of the secondary lipid-binding site. Again, this is in good agreement with our hypothesis that the D-helix of repeat III has to refold for proper calcium binding.

We would like to quantitatively determine the conformational changes that occur upon calcium and/or membrane binding using spectroscopic techniques in the near future.

Membrane Interaction of the Annexin 1 N-terminal Domain

How does annexin 1 promote membrane aggregation? In order to induce membrane aggregation or even fusion, the protein has to bring two membranes in close contact. This could be accomplished by either multimerization (i.e., dimer formation) or by providing two separate membrane-binding sites per molecule.

Our proposed mechanism of the annexin 1-annexin 1-induced aggregation of membranes by monomers utilizing two distinct membrane binding sites does not only stem from our full-length annexin 1 structure, but it is also based on research reported by other laboratories. In 1992, Meers and co-workers realized for the first time, that annexin 1 monomers might be able to contact two bilayers simultaneously and thus induce aggregation.[9] Reutelingsperger's group published two papers in 1993 revealing the N-terminus of annexin 1 as the part of the protein responsible for membrane aggregation: a chimera comprised of the core of annexin 5, which is not able to promote vesicle aggregation itself, and the N-terminal domain of annexin 1 has been shown to induce membrane aggregation.[15,16] Other researchers observed a secondary lipid-binding site of annexin 1 after the primary binding to negatively charged phospholipids in the presence of calcium ions. This secondary binding site does not exhibit lipid specificity in contrast to the primary binding site.[13,20] Again, the N-terminal domain seems to play a role in this secondary binding, because after primary calcium-dependent binding to membranes the N-terminus becomes exposed as shown by controlled proteolysis experiments. Proteolysis of annexin 1 bound to phosphatidylserine-vesicles was almost complete after one minute, whereas about 30 minutes were required to cleave annexin 1 in solution.[17,19]

Combining the results from these publications, it seems likely that annexin 1 binds to membranes containing negatively charged phospholipids first. This binding event is calcium-dependent, and involves the convex site of the molecule using calcium ions as bridges between the annexin and the bilayer. Upon binding to the first membrane, a conformational change on the concave site of the protein is induced whereupon the amphipathic N-terminal domain of annexin 1 becomes solvent (and protease!)-accessible. More support of this hypothesis arises from studies using x-ray reflectivity measurements of annexin 1 bound to 1-palmitoyl-2-oleoyl-*sn*-glycerophosphoserine/1-palmitoyl-2-oleoyl-*sn*-glycerophosphoethanolamine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPS/POPE/POPC, 2:5:2 in mol ratio) monolayers. These measurements confirm that annexin 1 forms a protein monolayer rather than a bilayer. In this monomeric form, annexin 1 is able to bind to POPC vesicles in a second binding event as shown by surface plasmon resonance measurements.[20] Additionally, cryo-electron microscopy showed two different types of junctions formed between annexin 1 and DOPG/DOPC liposomes: based on their relative thickness, one is thought to represent a single layer of annexin 1 monomers, the other annexin 1 dimers between the membranes.[32]

Taking into account all these results and our x-ray structure of full-length annexin 1 in the absence of calcium resulted in the model shown in Figure 5 in which we propose that the secondary membrane-binding site is in fact the N-terminal domain. X-ray crystallography revealed that the first 26 amino acid residues of the N-terminal domain fold into an α-helix and moreover, the first 12 amino acid residues form an amphipathic α-helix (see Fig. 3).[24,26] A peptide comprising the first 26 amino acid residues of annexin 1 has been shown by NMR analysis to form an α-helix in 10 mM SDS and 50%TFE/water, therefore, it is thought to be able to interact with membranes itself.[21] Generally, peptides forming amphipathic helices have been shown to interact with lipid bilayers (membranes) and they usually reside at the membrane interface. Examples include model ion channel peptides,[34] general model peptides folding into amphipathic helices,[35] model peptides for class A amphipathic helices of apolipoprotein A-1[36] and melittin (the 26-residue membrane-lytic peptide of the European honeybee).[37] Similar to annexin 1, amphipathic α-helices at the N-terminus have also been discovered as membrane

binding domains in RGS4, a GTPAse-activating protein for G protein α subunits, and prostaglandin endoperoxide synthase-1 and synthase-2 isozymes.[38,39]

Conclusions

The x-ray structure of full-length annexin 1 in the absence of calcium ions provided insight into a possible membrane binding and aggregation mechanism by annexin 1. As supported by other experimental data, annexin 1 could bind to the first membrane composed of negatively charged phospholipids in a calcium-dependent fashion, and after a conformational change releasing the N-terminal domain from the core, a second membrane binding event could take place. The first 12 amino acid residues of the N-terminal domain of annexin 1 form an amphipathic helix that could interact with a second bilayer. To gather further support for this two-step reaction mechanism we have to show firstly that the N-terminal helix is expelled from the protein core upon calcium- and/or membrane-binding. Secondly, we have to show that a peptide comprising the first 12–26 amino acid residues of the N-terminal is able to interact with phospholipid membranes as a pure peptide or included in a fusion protein. To investigate this two-step membrane aggregation mechanism of annexin 1 and to include other annexins that have been shown to induce membrane aggregation (i.e., annexins 2 and 7) will be the goal for our future research.

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