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Three-dimensional structure of annexins

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Abstract. Annexins constitute a family of structurally related calcium- and phospholipid-binding proteins whose molecular structure has been investigated in detail in the crystalline and membrane-bound form. Their polypeptide chain is folded into four or eight α-helical domains of similar structure with a central hydrophilic pore. Bound to phospholipid membranes, the four-domain arrangement of the annexin molecule is conserved. A peripheral binding mode has been well documented by electron microscopy and a variety of other techniques.
Key words. Annexin; crystal structure; electron microscopy; interfacial membrane proteins; ion channel.

Introduction

Annexins belong to a family of structurally related proteins which bind to negatively charged phospholipids in a calcium-dependent manner. Annexins are clearly distinct from the well-known 'EF hand' proteins such as calmodulin and troponin C, in the sequence and geometry of their calcium-binding sites as well as their affinity for calcium. Although the biochemical properties of annexins have been extensively investigated and their molecular structure, in crystalline and membrane-bound forms, has been elucidated in detail, their in vivo function still remains unclear. Annexins have been implicated in membrane-trafficking processes such as endo- and exocytosis, anti-inflammation due to the inhibition of phospholipase A_2, blood coagulation, organization of the cytoskeleton, cell differentiation, cell proliferation, mitogenic signal transduction and voltage-regulated ion channelling processes (for reviews see [1–6]).

Annexins are composed of four (or eight in the case of annexin VI) homologous repeats of about 70 amino acids. The repeats share a sequence identity of 25–35% between each other [7] and of 45–55% among different annexins [8]. They all harbour a characteristic calcium- and phospholipid-binding site within a 17 amino acid consensus sequence termed the 'endonexin fold' [9, 10]. In contrast to the homologous protein 'core', the annexins' N-termini are diverse in sequence and length, ranging from 11 to 196 residues. This N-terminal tail is subjected to various post-translational modifications and is thought to be the regulatory domain which may confer specific functions upon each annexin member.

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Crystal structures

The first annexin to be characterized by its three-dimensional structure was human annexin V in 1990 [11] (fig. 1). So far the molecular structure of human annexin V has been solved in various crystal forms [12–14], as well as the structures of chicken [15] and rat annexin V [16]. Furthermore, the crystal structures of human annexin III [17], human annexin VI [18], annexin XII from *Hydra vulgaris* [19] and the structures of N-terminally truncated forms of human annexin I [20], human annexin II [21] and annexin VII from *Dictyostelium discoideum* [51] have been determined.

All annexin structures reveal the same protein topology (fig. 2), as expected from the high primary sequence homology. In agreement with circular dichroism [22, 23], annexins are shown to be almost entirely α-helical. With the exception of annexin VI, the polypeptide chain is folded into four compact domains of similar structure (labelled from I to IV) corresponding to the four homologous amino acid repeats. Each domain consists of five α-helices (labelled from A to E) wound into less than two turns of a right-handed superhelix. Four of the helices are oriented approximately (anti)-parallel to each other whereas the fifth helix lies almost perpendicular to them. The domains I and II, and III and IV, respectively, are connected by short interhelical turns, whereas the connector between the domains I and II is extended.

The four domains are arranged in an almost planar cyclic array. The molecule has an overall flat, slightly curved shape with a convex and a concave side. All calcium ions defined in the various crystal structures are located on the convex face, whereas the N- and C-terminus is placed on the opposite concave side. The domains II and III, and I and IV, respectively, have tight contacts mediated by hydrophobic residues, thus generating two modules with approximately twofold symmetry. Both modules interact less tightly, mostly by

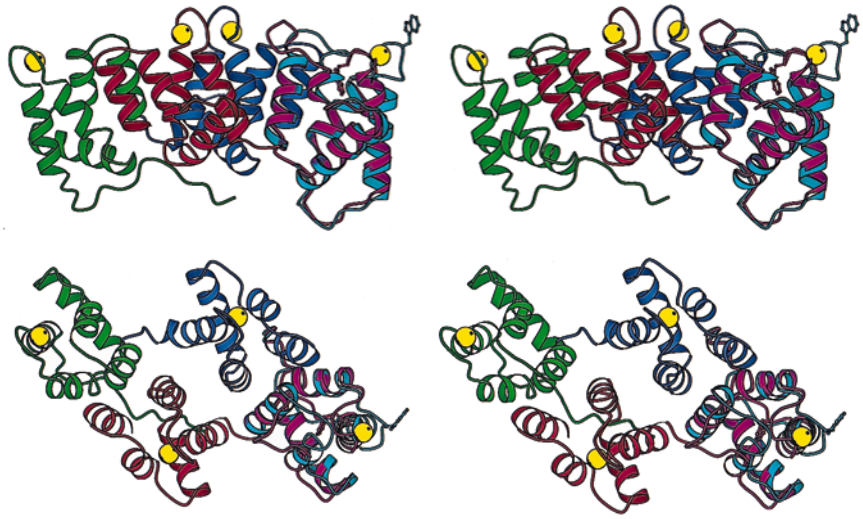

Figure 1. Stereo ribbon plots of human annexin V viewing (top) from the molecule side and (bottom) from the convex side harbouring the highly conserved calcium-binding sites. The high- [25] and low-calcium forms [30] are superimposed, showing the conformational change in domain III. Calcium ions are depicted as yellow spheres and the four domains are indicated in different colours: domain I, green; domain II, dark blue; domain III, light blue (high-calcium form) and pink (low-calcium form); domain IV, red. The side chain of Trp-187 is shown in ball-and-stick representation. Graphics were created with MOLSCRIPT [50].

polar and charged residues and are related by another twofold local dyad. This axis marks the centre of the molecule and a very prominent hydrophilic pore. The framework of this funnel-shaped pore is formed by a four-helix bundle coated with highly conserved charged amino acid residues. Buried well-ordered water molecules in the structure are located predominantly within this channel. Single channel measurements on annexin V have identified this pore to be the calcium-selective ion conduction pathway [24–26]. Ion channel activity was also reported for annexins I, II, VI and VII [18, 27–29]. For the ion channelling process, side chain rearrangement at least within the rather narrow pore would be required, including the two salt bridges invariant among the annexins.

Recently, the crystal structure of human annexin VI, the only annexin composed of eight amino acid repeats, has been determined [18]. The molecule consists of two similar halves, closely resembling other annexin structures (fig. 3). The halves are arranged perpendicular to each other connected by a 49 amino acid α-helical segment. Therefore the calcium- and membrane-binding sites are not located in the same plane, a rather unexpected feature of the membrane binding properties of the protein. The two halves cohere by interactions of domains III and IV of the first half with domains VII and VIII of the second half, thereby burying nearly 11% of the total accessible molecular surface. Their contact is stabilized by four salt bridges and the interaction with the connector helix which packs tightly to helix C of domain VII and also mediates hydrophilic contacts to the first half.

A further interesting structural feature was presented in the crystal structure of Hydra annexin XII [19]. A protein hexamer obeying 3-2 symmetry was found with the shape of a concave disk about 100 Å in diameter and 70 Å thick. Two annexin XII trimers, similar to trimers in crystals of annexin V [11, 30] are joined face-to-face by their convex sides. Six intermolecular calcium ions are involved in hexamer formation and an additional 18 calcium ions are located on the perimeter of the disk.

In contrast, a protein dimer was obtained in crystals of *Dictyostelium* annexin VII. As is annexin XII, both molecules interact with their convex sides, but in this case the dimer is preferentially stabilized by hydrophobic contacts in the absence of calcium [51]. For both annexin VII and XII, a biological role of the observed dimer and hexamer remains to be discovered.

Calcium-binding sites

Most annexins have been crystallized at millimolar calcium concentrations and the metal ions were found to be well defined in the molecular structures. Exceptions are the hexagonal crystal form of human annexin V in which the calcium-binding sites are not well ordered [11], and the calcium-free structures of human annexin VI [18] and annexin VII from *Dictyostelium discoideum* [51].

The characteristic calcium-binding sites of the annexins are found within the 'endonexin fold' in each of the four domains. The calcium is bound to a loop between helices A and B with the consensus sequence G-X-G-T-{38}-(D/E) in which the glycines permit a tight loop geometry. The calcium is coordinated by three main chain carbonyl oxygen atoms of the loop, a bidentate carboxylate group from an acidic side chain 38 residues down the

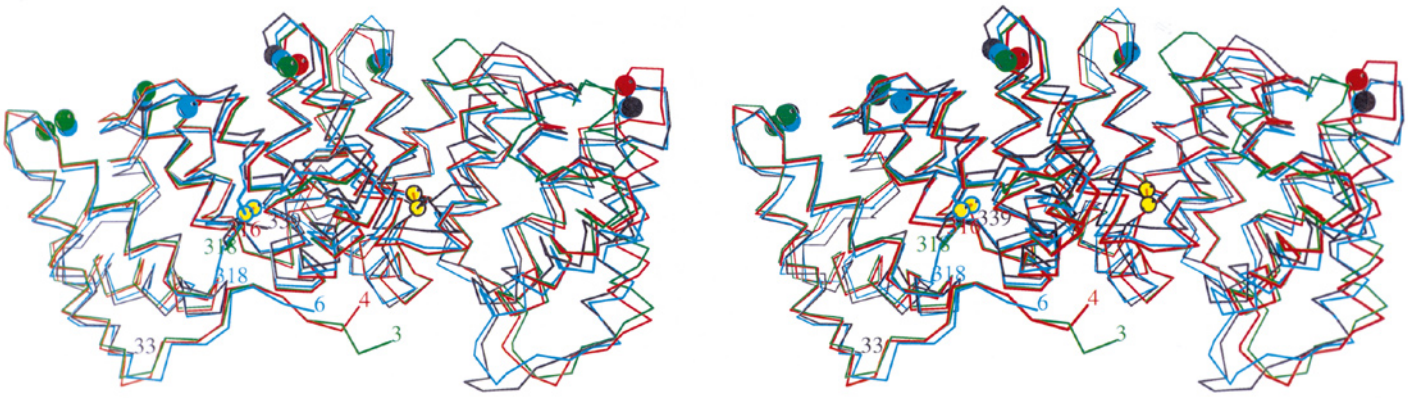

Figure 2. Overlay of the structures of annexin I (blue), II (black), and V (green: low-calcium form; red: high-calcium form) seen from the molecule side (stereo view). The calcium ions are shown as spheres in the colours of the associated proteins. The cystine 297-316 of annexin I and cystine 133-262 of annexin II are drawn as yellow spheres (program MOLSCRIPT [50]).

sequence in the helix D-E loop, and additional water molecules or a sulphate ion from the precipitating agent, respectively. The coordination sphere is a pentagonal bipyramid with a main chain carbonyl and a water molecule at its vertices. The calcium oxygen distances are between 2.4 and 2.7 Å. These characteristic calcium-binding sites bind calcium with K_d values in the micro- to millimolar range and have been termed type II binding sites [12] in order to distinguish them from the typical 'E-F hand' sites. 'E-F hands' show a higher calcium affinity and a different structure, presenting a loop of 12–14 amino acid residues that contributes 6–8 oxygen atoms for calcium binding and is flanked by two α-helices [31]. The annexins' type II sites are closely related to the calcium binding site in the catalytic centre of phospholipase A_2 which shows a comparable calcium affinity but has placed the coordinating bidentate acidic side chain only 16 residues away [32]. The type II binding sites have been proposed to be phospholipid binding sites as well, and recent crystal structures of calcium-annexin V complexes with phospholipid polar heads have confirmed the 'calcium bridging' mechanism for annexins as interfacial membrane proteins [33]. Upon membrane binding a water molecule or sulphate ion from the apical position of the calcium coordination sphere can be replaced by a phosphoryl oxygen of the phospholipid backbone. Analysis of the complexes with glycerophosphoserine and glycerophosphoethanolamine revealed highly specific interactions between the protein and the serine in contrast to the ethanolamine head group as well as coordination of the serine carboxylate oxygen with a second calcium ion which may explain the strong preference of annexins for phosphatidyl serine in vivo [33].

In all calcium-loaded annexin structures a second type of calcium binding site, called type III [20], has been found. The calcium is bound to the helix D-E loop by one or two main chain carbonyl oxygen atoms, a bidentate carboxylate from the E helix and several water molecules. This type of binding site displays lower affinity to calcium but higher affinity to lanthanum ions and is therefore called a lanthanum binding site [11].

Two different conformations have been observed for the third repeat of annexin V whose calcium-binding sequence is G-E-L-K-W-G-T-{38}-E and therefore slightly deviates from the other repeats (fig. 1a, b). Crystallization under low calcium concentrations showed a structure with three canonically bound calcium ions in domains I, II and IV [13, 15, 30]. Under high calcium concentrations a new crystal form exists in which the normally buried tryptophan side chain in the helix A-B loop of domain III becomes exposed to the molecular surface and is thereby moved about a distance of 18 Å [14, 16, 25]. Due to this drastic conformational change a new calcium binding site within domain III is formed, structurally very similar to the canonical sites in the other three repeats. In conclusion, the buried or exposed conformation within this domain depends on crystal packing in a highly sensitive calcium-dependent manner. The same conformational change has been observed in solution by fluorescence studies in the presence of calcium and phospholipids, suggesting a direct contact between the exposed tryptophan and the ester-bond region of the membrane [34, 35]. Annexins III and VI also contain a tryptophan in the A-B loop of the third domain. But in contrast to annexin V, this tryptophan side chain is always solvent-exposed, even in the absence of calcium in solution [17, 18].

Annexins I and II display the unique calcium binding sequence X-K-G-V-{38}-A in domain I which deviates from other annexins and lacks the acidic amino acid side chain for bidentate coordination [20, 21]. Nevertheless, both annexins are able to bind calcium at this binding site, but in a rather different way with a neighbouring glutamate as a bidentate ligand.

Membrane-bound annexins

Annexins are amphipathic proteins, different from soluble and integral membrane proteins. Their binding to

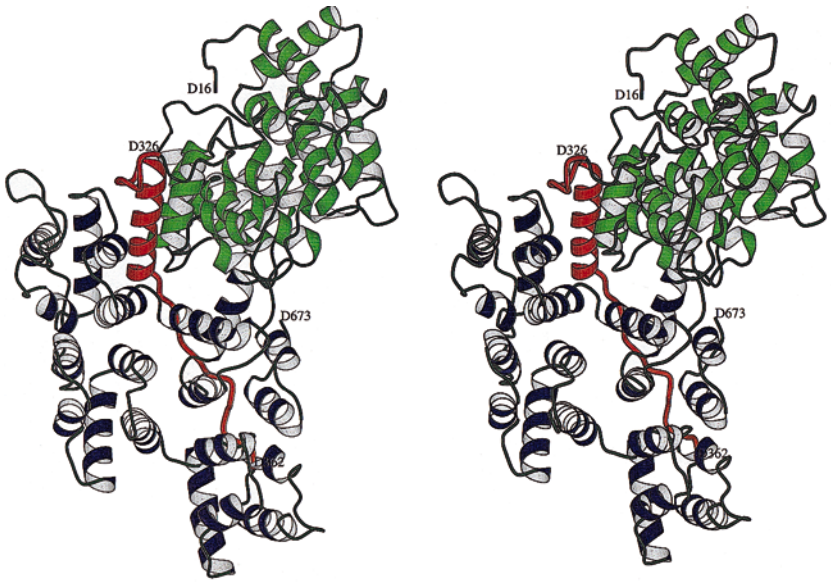

Figure 3. Stereo images of a ribbon plot of human annexin VI composed of two four-repeat halves [18]. The N-terminal half is depicted in green, the C-terminal half in blue and the connector in red. The view is onto the convex, membrane binding face of the C-terminal half (program MOLSCRIPT [50]). (Reprinted with permission from: Benz et al. (1996) The structure of recombinant human annexin VI in crystals and membrane-bound. J. Molec. Biol. **260:** 638–643, Academic Press, London.)

phospholipids is calcium-dependent and rapidly reversible by addition of calcium chelators such as EDTA. Although the occurrence of EDTA resistant forms has been described, their distribution and structure are not yet understood.

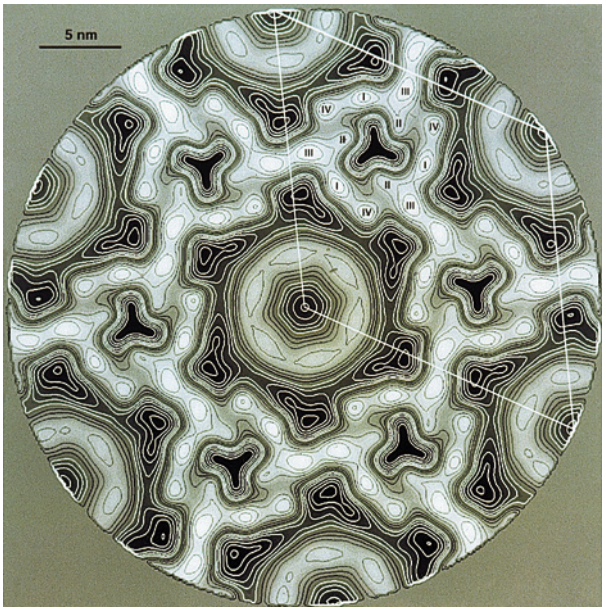

Figure 4. Electron micrographs of membrane-bound human annexin V. p6-Symmetry was imposed on the projections of the two-dimensional, negatively stained crystals with contour-lines and the domain assignment added. The unit cell is outlined by the white diamond and has a periodicity of about 18 nm. It contains two ordered trimers at the triads and an additional translationally and rotationally disordered trimer at the central hexad. (Reprinted with permission from: Voges et al. (1994) Three-dimensional structure of membrane-bound annexin V. A correlative electron microscopy-X-ray crystallography study. J. Molec. Biol. **238:** 199–213, Academic Press, London.)

Self-association on membrane surfaces has been described for various annexins. Annexin V forms calcium-induced trimers, hexamers and higher aggregates on phospholipid membranes as observed by chemical crosslinking experiments [36]. The membrane-bound structures of annexin V [37–41] and annexin VI [42, 43] have been investigated by electron microscopy. Negatively stained two-dimensional crystals containing trimers of annexin V have been analysed at 8 Å [40] and 17 Å resolution [41] (fig. 4) and image processing of recorded tilt series allowed the three-dimensional structure of the annexin V-membrane complex to be reconstructed [41]. A comparison with the high resolution crystal structure [11, 30] shows that the domain structure is highly conserved on the membrane, but the relative orientation of the modules (II/III) and (I/IV) is slightly changed so that the calcium-binding sites in all four domains become coplanar to the membrane. The thickness of the molecule obtained in the three-dimensional reconstruction corresponds well with the thickness of the high resolution crystal structure indicating a peripheral binding of annexin V without substantial penetration of the membrane [41]. This observation is in accordance with other experimental results clearly demonstrating surface binding of protein monolayers on phospholipid membranes: annexin V binding to lipid bilayers was studied by ellipsometry thereby observing a mass coverage that corresponds to a single protein layer [44]. Low-angle neutron scattering on small unilamellar vesicles showed an increase of the radius of gyration after addition of annexin V in the presence of calcium, consistent with the formation of a protein monolayer

shell of 3.5 nm thickness with little or no protein insertion [45]. ^{1}H-NMR T_1 relaxation measurements on small unilamellar vesicles showed no influence on the hydrocarbon chain segmental motions upon calcium-dependent annexin V binding, while ^{31}P-NMR spectra revealed a shift in the outer- and inner-leaflet phosphoryl head groups [46]. These data suggest that protein binding occurs only peripherally but also affects the environment of the inner vesicular surface, probably due to a protein-induced change in vesicle morphology [46]. Recently, electron microscopic studies of two-dimensional crystals of annexin VI were performed at 16 Å resolution [18]. Whereas in the crystals both half molecules are arranged perpendicular to each other, both halves are coplanar bound to the membrane as expected for optimal binding. They are oriented relative to the membrane plane with their calcium binding sites facing the lipids. Upon membrane binding the two half molecules reorientate relative to each other by about 90°, suggesting a high flexibility of the α-helical connector which may facilitate membrane fusion and vesicle aggregation associated with annexin VI [47].

Ion channel activity

Whereas for several other water-soluble channel-forming proteins, integration into lipid membranes inducing profound structural changes have been proposed, all experimental data available to date confirm that annexins are peripherally bound. A molecular model therefore has to be established to explain how ions are translocated through the ternary annexin-calcium-membrane complex. Mutagenesis studies in combination with single-channel measurements have identified the central hydrophilic pore as the ion conduction pathway. Disruption of one of the invariant salt bridges within the annexin V pore led to a mutant (Glu->112Gly) which has lost voltage-dependent gating of the channel and selectivity for calcium over monovalent cations in comparison to the wildtype [26]. A further mutation within the pore (Glu95->Ser) showed an analogous selectivity change, thus suggesting that the selectivity filter of the channel is located within the hydrophilic four-helix bundle [25]. In contrast, mutations on the molecular surface showed no alterations in voltage-gating and selectivity of the channel [24].

In order to explain the pore formation in the active channel complex a phenomenon of 'microscopic electroporation' has been suggested for annexin V. This model is based on electrostatic calculations [48] and is consistent with the well-documented peripheral membrane interaction. For annexin VII a refolding of the molecule into a 'TIM barrel' structure with a complete penetration of the phospholipid bilayer has been proposed [49], whereas for annexin XII a complete insertion of the protein hexamer has been discussed [19]. In summary, the molecular picture of the annexins' ion conducting state is still controversial, although an equivalent mechanism for all annexins can be expected. Therefore, elucidation of the ion channelling process is still an important task.

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