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A model of the structure of human annexin VI bound to lipid monolayers

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Annexin VI is an eight repeat member of the annexin family of proteins which are both water soluble and bind to negatively charged phospholipids in a calcium-dependent manner. Here we present a model for annexin VI based on fitting the three-dimensional structure of two annexin V molecules (Huber (1990) EMBO J. 9, 3867–3874) to the two-dimensional stain-excluding density of lipid-bound annexin VI (Newman (1989) J. Mol. Biol. 206, 213–219). Both annexin VI lobes could only be fitted with their convex faces closest to the lipid monolayer. This supports the hypothesis that annexin–lipid binding is mediated by the interaction between calcium bound to the loops protruding from the convex protein surface and phospholipid headgroups.

Annexin; Protein structure; Membrane; Electron microscopy; Phospholipid monolayer

1. INTRODUCTION

The annexin family comprises at least ten different proteins, all of which are water-soluble and bind to negative phospholipids in a calcium-dependent manner [3–6]. They are widely distributed, being found in plants, insects and animals, which suggests that they have an important basic biological function. All members share a conserved 70 amino acid repeating unit and the family can be further subdivided into four-repeat- and eight-repeat-containing proteins [7]. A number of possible physiological functions have been suggested for different members [8] including regulation of inflammation via phospholipase A2 inhibition, regulation of blood coagulation, cytoskeletal organisation, membrane-membrane fusion and exocytosis, signal transduction, cell growth, differentiation and immunomodulation. At the molecular level, annexins V and VII have been shown to act in vitro as voltage-gated cation channels with a high selectivity for calcium [9–11].

The single-crystal X-ray structure of human placental annexin V has been determined to 2.0 Å resolution [1,12]. The molecule is folded into a planar cyclic arrangement of four domains, corresponding to the four sequence repeats, with each domain comprising five α-helices wound into a right-handed superhelix. The molecule appears asymmetric, with all of the calcium binding sites on the convex face of the molecule, which forms the proposed lipid-binding surface [12–14]. Calcium is bound to three of the four potential calcium binding loops and to a further two low-affinity sites [13]. Through the centre of the molecule, along the central intramolecular 2-fold axis, lies a channel surrounded by α-helices and lined with many charged amino acid side chains. Huber and colleagues have suggested that the initial binding of annexin V to phospholipids is regulated by the electrostatic potential of the protein [15]. Furthermore, they have shown that a strong local gradient of electrostatic potential could exist at the protein–membrane interface, especially at the calcium binding loops, and that a membrane pore may be generated by electroporation [12,15]. The observed specificity of ion conduction and regulation is suggested to reside within the pore of the protein. The rearrangement of the salt bridges: Arg*271*-Glu*112* to Arg*271*-Glu*95* and Glu*121*-Arg*276* to Glu*121*-Arg*117* would open the protein pore without a large change in conformation and electrostatic free energy [15].

Annexin V bound to dimyristoylphosphatidylethanolamine (DMPE) lipid monolayers [16] (incorrectly assigned annexin IV) and dioleoylphosphatidylserine, dioleoylphosphatidylcholine mixed lipid layers [17] has been visualised at low resolution by electron microscopy methods. Two-dimensional projected stain excluding density maps revealed triskelion-like motifs with p6 [16] and p3 symmetry [17]. A superposition of the three-dimensional structure and the two-dimensional stain-excluding density for the second case [14, 17] showed close correspondence indicating that the three-dimensional structure of annexin V does not undergo significant conformational changes upon binding to lipids. Moreover, a similar packing was observed for the three-

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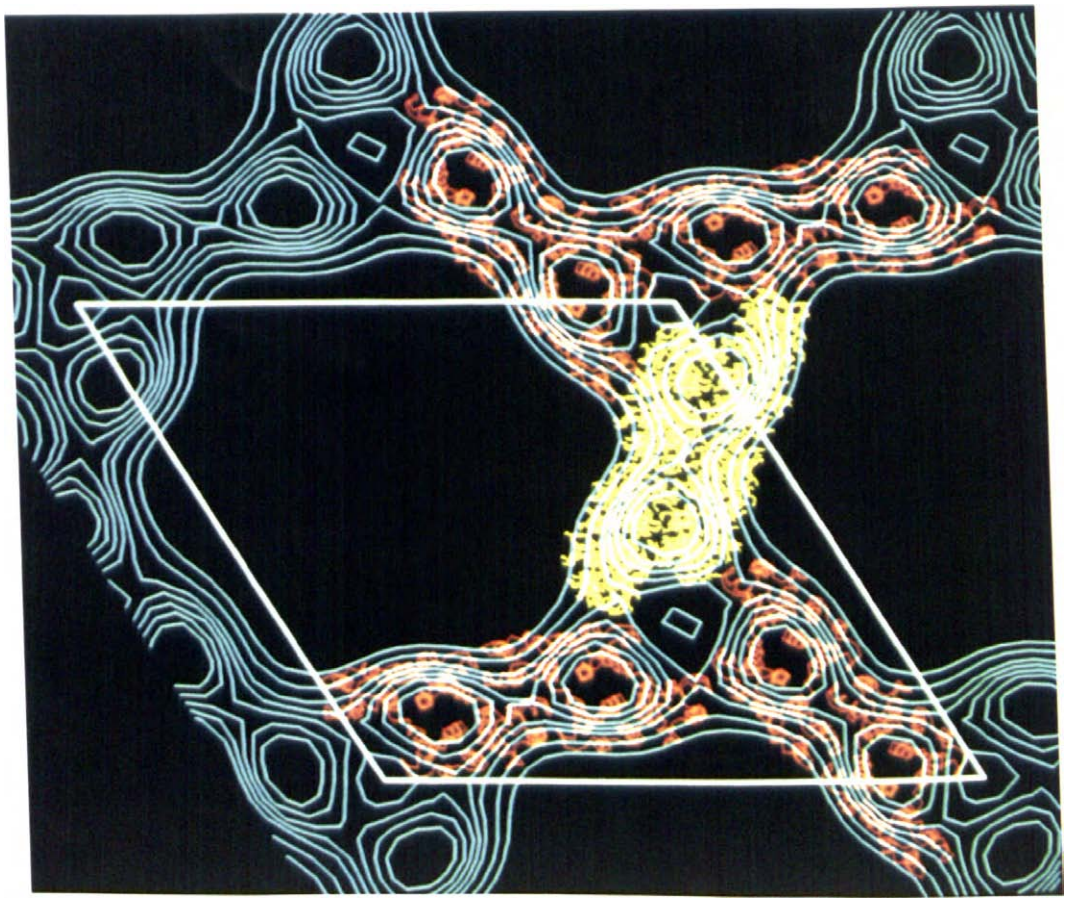

Fig. 1. Superposition of the bilobal annexin VI model, built from two annexin V molecules, on the annexin VI stain-excluding density. The model is viewed from the concave side of the protein along the central pores towards the membrane. Handedness was checked by using grids with a known orientation. The map (pale blue) is contoured at levels of 1/6 sigma starting at 0. Five annexin VI molecules are shown packing around two of the three 3-fold axes of the unit cell. The central molecule (yellow) is represented with all atoms and the others (red) by C-α atoms only.

dimensional structure (p3 sub-lattice of R3) and the stain-excluding density. The superposition also showed the putative calcium channel within the annexin V molecule to be perpendicular to the membrane, with domain three from each of the three molecules forming a central 3-fold symmetrical contact. It was not possible to define the orientation of the annexin V molecule relative to the lipid layer, as lipid multi-layers were analysed. However, a model which positions the calcium binding loops of annexin V near the phospholipid headgroups is supported by many observations including the calcium dependence of phospholipid binding.

Annexin VI is an eight-repeat annexin comprising two four-repeat modules with each module homologous to the single four repeat annexin V molecule (sequence identity 49% for repeats 1 to 4, 47% for repeats 5 to 8; [7]). Secondary structure predictions suggest the presence of α-helices in positions homologous to annexin V [7]. Therefore, annexin VI can be viewed as a covalent dimer of annexin V. Annexin VI has also been crystallised on lipid monolayers of DMPE [2]. The crystals displayed a hexagonal lattice and the projected stain excluding density at 50 Å resolution revealed a trimeric packing, with the asymmetric unit containing one annexin VI molecule (Fig. 1). The two similar stain-excluding density peaks (50–55 Å diameter) inside the asymmetric unit are likely to correspond to the two lobes of annexin VI. Although the space group for the annexin VI-lipid complex was planar group p3, with a phase residual of 11.13° for the symmetry equivalents, the imposition of p6 symmetry produced a phase residual of 15.92°. This suggests the presence of a non-crystallographic 2-fold axis within the asymmetric unit of the p3 cell. The non-crystallographic nature of the symmetry is also indicated by the slightly different dimensions of the holes at the 3-fold axes where the molecules interact and in the marginally different orientations of the peaks (Fig. 1). An EM study of sectioned microcrystals of annexin VI [18] showed the molecule to be peanut-shaped in projection and of similar dimensions to that observed bound to lipid. However, the absence of an apparent 2-fold axis in the observed peanut-shape suggests that the lipid-bound and crystalline annexin VI are either viewed from a different orientation or have

different structures. Here we have used the structure of annexin V [1,12] and the projected stain-excluding density map of annexin VI bound to lipid monolayers [2] to examine possible models for annexin VI.

2. MATERIALS AND METHODS

Stain-excluding density maps were calculated using the fast Fourier transform program algorithm [19] as implemented in the CCP4 program FFT [20]. Model building was carried out using the interactive computer graphics program FRODO [21], as modified by P. Evans. Lattice contacts were calculated for the model for atoms less than 4.0 Å apart in 9 neighbouring unit cells by the program MODEL (H.P.C. Driessen and P. Lindley, unpublished), and were examined further using FRODO. For relevant residues amino acid substitutions between annexin V and VI were taken into account. Contact sites were classified depending on the type of interactions involved; hydrophilic for hydrogen bonds and ions pairs; hydrophobic for carbon-carbon interactions between Ile, Met, Phe, Pro, Trp, Tyr and Val. The accessible surface area was calculated by the method of Kabsch and Sander [22].

3. RESULTS AND DISCUSSION

Annexin V has been shown to bind to lipids, with its pore perpendicular to the membrane, in plane group p3 with a cell length of 94 Å [17]. As the cell length for annexin VI is 178.2 Å [2], which is approximately twice the size, a similar orientation may be expected. A planar projection enclosing all the atoms of the three-dimensional structure of annexin V seen along its central channel can be described as a rhombus with side of ca. 54 Å (Fig. 2). We took two annexin V molecules, in the above orientation, and centred them on the peaks in the density of the annexin VI map ensuring sensible intermolecular contacts. The two molecules are related by a lateral translation and because of the presence of a central pseudo 2-fold axis in each annexin V molecule, a third pseudo 2-fold axis was automatically generated between them. The projection of the resulting planar bicyclic model shown in Fig. 1 can be viewed as a parallelogram with sides of 100 Å by 54 Å comprising two partially overlapping rhombuses (Fig. 2). The distance between the two crystallographic 3-fold axes around which the proteins interact is 102.9 Å and therefore only the short diagonal of the parallelogram can be fitted into the density between the two axes. The annexin V molecules only fit the density if projected from the concave side. Because of the shape of each rhombus, it is not possible to fit the two molecules upside down with their pores still perpendicular to the membrane without positioning part of the molecules on the 3-fold axes or causing intermolecular clashes (Fig. 3). To optimise the fit while keeping the contact areas acceptable, both molecules were rotated slightly around their intra-lobal 2-fold axes giving a deviation of 3° for the inter-lobal 2-fold axis. The presented model fits the stain excluding density remarkably well (Fig. 1).

The connectivity for annexin VI built from two molecules of annexin V can be described as the linear sequence of domains 1 to 8. Domains 1 to 4 (lobe 1) correspond to domains 1 to 4 of annexin V molecule 1 while domains 5 to 8 (lobe 2) correspond to domains 1 to 4 of annexin V molecule 2. In the presented model the connectivity of the individual lobes is repeated by the applied translation bringing domains 4 and 5 and 3 and 6 in close proximity, while domains 1 and 7 are maximally far apart (Fig. 2). The pseudo 2-fold axis of the p3 cell is therefore generated by the presence of the central intramolecular pseudo 2-fold axis in each annexin VI lobe. The alternative translational model which places domains 3 and 5 maximally far apart would require a connecting peptide having a length of almost 100 Å. The number of residues between the last and first helices in domains 4 and 5, respectively, including the linker peptide (326–347) is 39. This would require an average distance per residue of 2.6 Å minimally, which could be possible, although secondary structure predictions suggest an α-helical conformation for residues (334–347), which if present makes this unlikely. The slight deviation of 3° around the inter-lobal 2-fold axis may accommodate this extra helix.

In principle it would be possible to construct two models where the two lobes are related by an actual rotation of 180°, which would not require the central pseudo 2-fold axis of the annexin V molecule. In the first

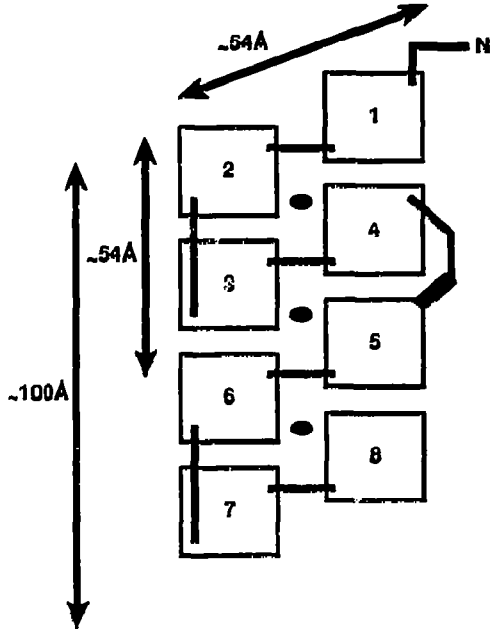

Fig. 2. Schematic representation of the planar bicyclic arrangement of the eight domains of annexin VI. The 2-fold axes between domains and lobes are indicated. The dimensions of the rhombus enclosing one lobe and the parallelogram enclosing two lobes are shown. The putative α-helix in the linking peptide between domains four and five is shown as a solid cylinder in an arbitrary position. The N terminus is labeled N. The orientation of the model is the same as in Fig. 1.

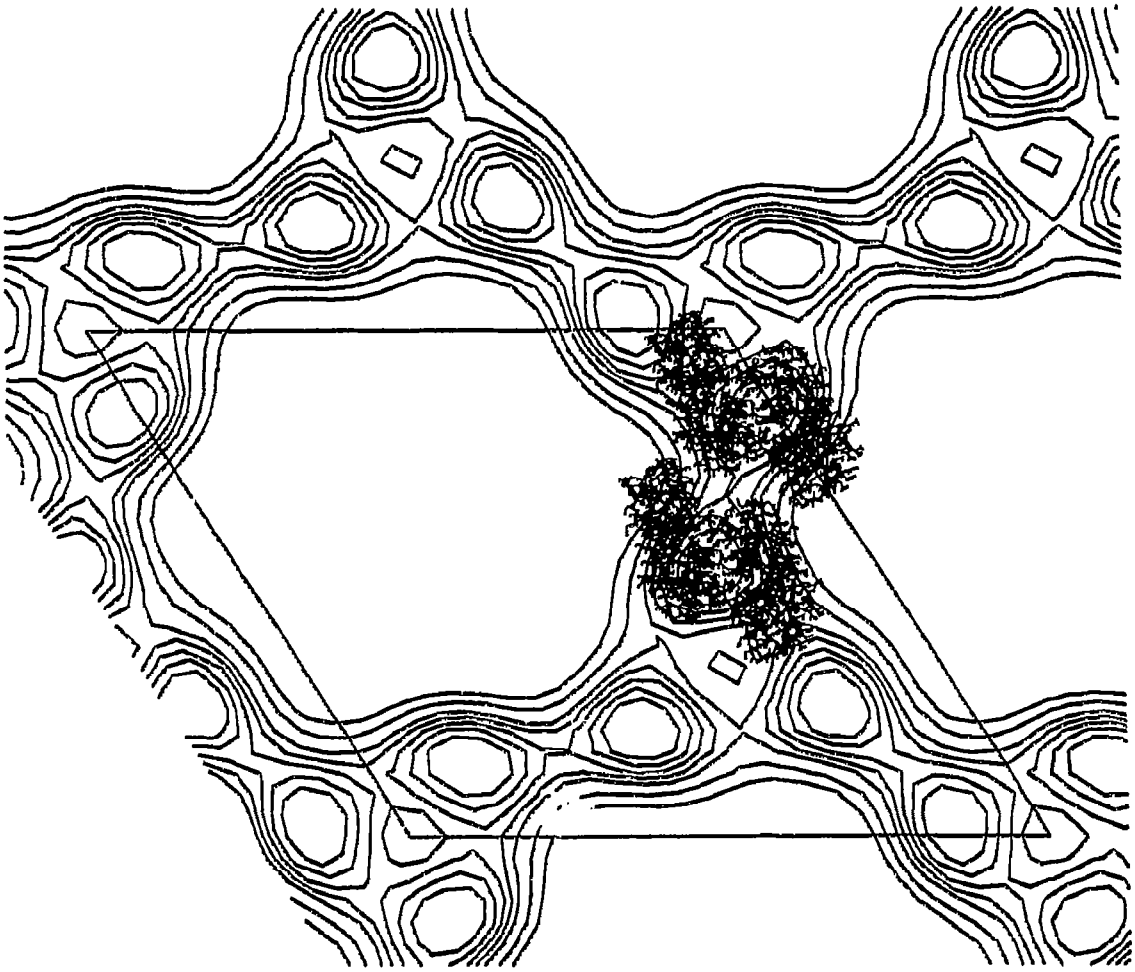

Fig. 3. Alternative superposition of the bilobal annexin VI model, built from two annexin V molecules (map parameters are as in Fig. 1). The model is now viewed from the convex side of the protein along the central pores towards the membrane. It is not possible to obtain a reasonable fit of the two molecules in this orientation.

case domains 1 and 5 will be maximally far apart, while 4 and 7 and 3 and 8 will be close together, and the contact zones at the two 3-fold axes would then be similar. However, the different sizes of the holes and orientations of the density peaks argue against this. Furthermore, this rotational model would require the connecting loop between domains 4 and 5 to have a length of the order of 100 Å making this also unlikely. The same situation exists for the alternative rotational model with domains 3 and 7 maximally far apart.

The annexin VI molecules are in close contact around an irregular hole (10–15 Å diameter) at the 3-fold axis on the origin (Fig. 1), with hydrophilic interactions between domains 1 and 2 of each molecule. Around the larger hole (ca. 28 Å diameter) at the intracellular 3-fold axis the molecules bind less extensively, and the intermolecular contacts are hydrophobic involving domains 7 and 8 from each molecule respectively. The inter-lobal contacts give a buried area of 1708 Å^2, constituting 11.4% of the surface of the two individual lobes. The residues which form the salt bridges of the proposed cation gate of annexin V are conserved in each lobe of annexin VI with the exception of 'Glu*95*' in lobe 2 and 'Glu*121*' in lobe 1 which are both glutamines making gates possible. Could the inter-lobal contact area represent a third hydrophilic pore, with the long connecting peptide regulating access to the gate in a similar way as the N terminus of annexin V? The inter-lobal contacts around the central pseudo 2-fold axis appear to be a mixture of polar and apolar, with the largest contact area between domains 3 and 5. Only 'Arg*117*' in domain 3 is completely conserved. Furthermore, the relative geometry of the α-helices in this area is different from that in the intra-lobal pore, as is the pore size. These three features therefore make a third gate of the annexin V type unlikely.

Mosser et al. [17] have listed the evidence for the association of annexins as trimers in near-physiological conditions. The observation that annexins often crystallise in hexagonal lattices [1,13,23] supports this idea. Although both annexins V and VI appear to bind to phospolipids as trimers, the packing of these trimers is

different. In the annexin V-membrane triskelion structure, the three molecules around the central 3-fold axis are in close contact [17]. However, in the annexin VI-membrane stain-excluding density, the molecules associate more loosely on a one-to-one basis around holes at two of the three 3-fold axes of the unit cell giving rise to two different kinds of trimers. The size of the holes in the annexin VI trimers is such that they are unlikely to be active cation channels. Although the existence of the pore [1,12,13] and the studies on annexin V binding to phospholipids [14] suggest that the calcium binding sites are closest to the lipid, there has been no direct evidence for this. The use of a lipid monolayer for the annexin VI studies, prepared using a hydrophobic trough [24], together with the presented model which has the convex side nearest the lipid, suggests that annexin-lipid binding is indeed mediated by interaction between the calcium binding loops and the phospholipid headgroups.

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