The calcium binding sites in human annexin V by crystal structure analysis at 2.0 Å resolution

Implications for membrane binding and calcium channel activity

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Crystal structure analysis and refinement at 2.0 Å resolution of a rhombohedral crystal form of human annexin V at high calcium concentration revealed a domain motion compared to the previously analysed hexagonal crystal form. Five calcium ions were located on the convex face of the molecule. Three strongly bound calciums are liganded at protruding interhelical loops and Asp or Glu residues in homologous positions in repeats I, II and IV. Five proteinaceous oxygens and one solvent molecule form the coordination polyhedron in each case. The unoccupied seventh site is suggested as the phospholipid headgroup binding site. Two more weakly bound sites were identified by lanthanum labelling. The structural features suggest that annexin V attaches with its convex face to membranes by specific calcium mediated interactions with at least three phospholipids. The adjacent membrane bilayer may thus become locally disordered and permeable to allow calcium inflow through the central polar channel of the molecule.

Annexin; Crystal structure; Calcium; Membrane; Channel

1. INTRODUCTION

Annexins are cytosolic calcium, membrane binding proteins widely distributed in different species and cell types. Their capacity to support membrane fusion, to inhibit blood coagulation and inflammation in a calcium-dependent manner was recognized and led to the isolation and characterization of several proteins by primary structures and functional properties. Despite diverse biological properties reflected in a confusing nomenclature, the amino acid sequences showed close similarities between them and suggested that they belong to a large family, named annexins (see [2–5] for reviews). The amino acid sequences show usually four, in annexin VI eight tandem repeats of about 80 residues length which are well conserved within the annexin family and a more variable N-terminal segment.

Annexins are members of a third class of amphipathic proteins distinct from soluble and integral membrane proteins. They are readily soluble in water and interact with membranes in a calcium dependent manner. Some members also form voltage-gated calcium specific channels when associated with membranes; a property of integral membrane proteins (see [6] for a review).

We studied annexin V from human placenta, a potent anticoagulant, by crystallography initially within our programme of structural studies of coagulation factors [7,8] and presented the crystal structure and molecular model of a hexagonal form of annexin V [1]. We showed that the molecule of 320 amino acid residues is almost entirely α-helical. The four tandem repeats are similarly folded into compact domains consisting of five α-helices wound into a right-handed super-helix. Four helices, A,B,D,E, have their axes approximately parallel, whereas the connecting helix C lies flat. The four domains are arranged in an almost planar, compact array such that domains II and III and I and IV, respectively, form tight molecules with approximate two-fold symmetry. A third local dyad (dyad A) relates modules (II, III) and (I,IV). All four domains have their molecular axes as defined by the axes of helices A,B,D,E similarly oriented. Dyad A marks the center of the molecule and a very prominent hydrophilic pore, which we associated with the calcium specific channel in annexin VII and V [6,9].

In our previous analysis we could not define the calcium binding sites which seem to be partly occupied only in the hexagonal crystal form.

The present study describes the analysis of the rhombohedral crystal form of human annexin V. (The nomenclature of structural elements of annexin V has been introduced by Huber et al. (1990) and is used here (see Fig. 3 in Huber et al., 1990). These crystals grow together with the hexagonal form but tolerate transfer to solutions with very high calcium and lanthanum concentrations. Under these conditions five calcium sites

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Table I

Crystallographic data for rhombohedral crystal form

Data collection

Derivative	Measurements		Completeness above 2.5σ		R_{SI}	R_{FSI}
	Total	Independent	∞–2.1 Å	2.1–2.0 Å		
NATI	76560	16323	0.73		0.107	0.052
CAPL	89835	20726	0.94	0.24	0.084	0.043
LAGP	52984	17503	0.82		0.093	0.057
EDLA	68033	19048	0.86	0.17	0.086	0.051

NATI = crystals in 3 M AS, 0.1 M Tris-HCl, pH 8.5, 1 mM CaCl$_2$
CAPL = crystals several hours in 2.5 M AS, 0.1 M Tris-HCl, pH 8.5, 50 mM Ca-acetate
LAGP = crystals overnight in 2.5 M AS, 0.1 M Tris-HCl, pH 8.5, 50 mM La(NO$_3$)$_3$
EDLA = crystals for several days in 3 M AS, 0.1 M Tris-HCl, pH 8.5, 10 mM EDTA, transferred overnight in 25 mM La(NO$_3$)$_3$, 2.5 M AS, 0.1 M Tris-HCl, pH 8.5
$R_{SI} = \Sigma(I-\langle I\rangle)/\Sigma I$, for all measurements
R_{FSI} = for averaged Friedel pairs

Structure solution

	Patterson search								
	Peak heights		σ	Rotation (°)			Translation (frac)		
	Maximum	Second		Z	X'	Y''	X	Y	Z
rotation(NATI)	16.6	13.7	1.6	37.0	181.0	0.0			
translation(NATI) function(1-2)	505	157	32				0.2895	0.5800	0.0000
rotation(CAPL) with complete molecule	2.0			37.0	181.0	−1.0			
with module (I,IV)	6.8			36.0	182.0	−2.0			
with module (II,III)	6.3			36.0	180.0	1.0			

	Refinement				
E(kcal)	σ(r)(Å)	σ(°)	R(8–2 Å)	active atoms	solvent
−2237	0.010	1.9	0.218	2470	66

$R = \Sigma \mid F_o - F_c \mid / \Sigma F_o$

were identified. The structural features suggest that the contact between protein and membrane is at the convex side of the protein mediated directly by calcium. A model for this interaction is proposed.

2. EXPERIMENTAL

The rhombohedral crystals (NATI) grow as described for the hexagonal form [1]. Their space group is R3 with lattice constants $a=b=99.6$ Å, $c=97.2$ Å, $\alpha=\beta=90°$ $\gamma=120°$. These crystals were transferred into a solution containing high concentrations of calcium or lanthanum (see Table I) whereby cracks develop immediately. They anneal within about 30 min. These crystals have slightly altered lattice constants ($a=b=99.6$ Å, $c=96.4$ Å, $\alpha=\beta=90°$, $\gamma=120°$) but excellent, even increased crystalline order (CAPL, LAGP). Some rhombohedral NATI crystals were soaked several days in 3 M ammonium sulfate, pH 8.5, containing 10 mM EDTA and then transferred into a solution with 50 mM La(NO$_3$)$_3$ (Table I). Also under these conditions, cracks develop and anneal (EDLA).

The rhombohedral crystal forms NATI and CAPL were analysed by Patterson search techniques [10–12] using the hexagonal model in its entirety and divided into the (II,III) and (I,IV) modules, respectively. In CAPL the latter model gave significantly higher correlation values and slightly different orientations compared to the complete model. The hexagonal and rhombohedral crystal structures were refined and the data of the CAPL crystal form are reported in Table I as they form the basis of the present discussion.

X-Ray intensities were measured with the FAST area detector (Nonius, Delft) on a Rigaku rotating generator, evaluated with MADNES [13] and scaled and absorption corrected [14]. Crystallographic calculations were mostly performed with PROTEIN [15]. Translation functions were calculated with E. Lattman's programmes modified by J. Deisenhofer and R. Huber. Refinement was carried out with EREF [16], model building and inspection with FRODO [17].

3. RESULTS

3.1. *Conformation changes*

Rotational search calculations had indicated a change by about 4° in relative orientation of the two modules in CAPL compared to the hexagonal form, which was confirmed by refinement. The models were superimposed by aligning modules (II, III). In Fig. 1 a significant relative motion of the modules is shown when viewed perpendicular to the polar molecular axis and approximately along the tilt axis. The calcium-rich form has a

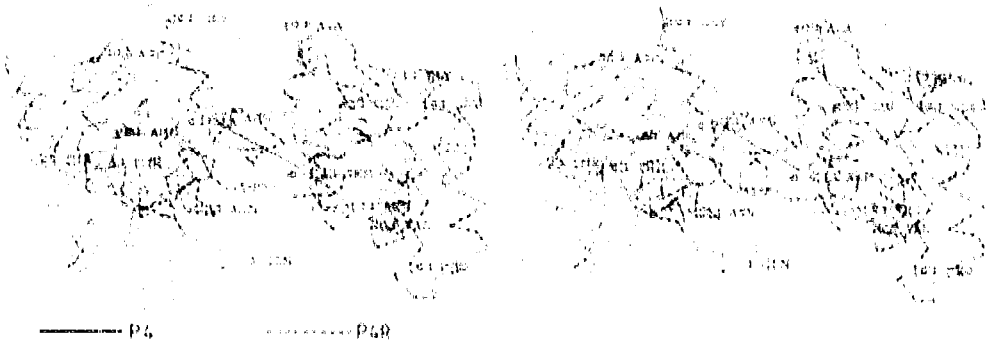


Fig. 1. C_α-chain tracing of the hexagonal crystal form (—) overlaid with the CAPL (high calcium) form. The modules (II, III) were superimposed. The CAPL form is more open by 'hinged domain motion' [Bennett and Huber, 1984]. The hinge is between helices IE and IIA and IIIE and IVA, respectively. The line and + , − signs mark the centres of the positive and negative electrostatic potential.

wider cleft separating the two modules and a more open central channel.

The structural change may be caused by the different crystal packing arrangements in the hexagonal and rhombohedral forms, but the rhombohedral NATI[a] form has been partially refined and seems to have a conformation similar to the hexagonal form. The conformation change is therefore a calcium effect. We point out that hexagonal crystals shatter when transferred into concentrated calcium or lanthanum solutions, while the rhombohedral crystals develop fissures which anneal. In these crystals (CAPL) the c-axis is contracted by 1.3% compared to NATI. In the rhombohedral crystals the calcium binding loops Ca1-3 are in lattice contacts and increased calcium ligation might cause slight packing rearrangements leading to an altered module tilt. Alternatively calcium ligation may directly induce the conformation change perhaps by repulsion of the protruding Ca1 and Ca2 loops which are 28 Å apart. Although the available data are insufficient to decide whether the calcium induced change of conformation is a molecular property or a crystal packing effect, they indicate a preferred mode of internal flexibility of the molecule.

3.2. *Calcium sites*

The primary goal of the study described was to define the calcium binding sites, which had not been identified in the hexagonal crystal form. The calcium sites Ca1,2,3 were identified by high electron density peaks in Fourier maps of CAPL and characteristic ligation by oxygens. Two more sites (Ca4,5) were indicated by lanthanum binding in the LAGP and EDLA derivates.

These five sites and their general locations are indicated in Fig. 2 superimposed onto a C_α-chain trace. They are located on the convex face of the molecule in protruding loop regions which will be shown in detail in Fig. 4 for Ca1 to Ca3.

Ca1 to Ca3 are bound to homologous segments in repeats I, II and IV, ligated to three carbonyl oxygens of the conserved (M,L)-K-G-(A,L)-G-T turns between helices A and B, to an Asp or Glu of the sequentially discontinuous, but spatially nearby helix D-E loop, and to a solvent molecule. The oxygen calcium distances are between 2.4 and 2.7 Å. The homologous segment in repeat 3 has a very different sequence -E-L-K-W-G-T and structure in annexin V and other annexins and does not bind calcium.

Fig. 3 shows the electron density map at Ca1 with the model fitted.

Fig. 4A-C show the Ca1-Ca3 binding sites in roughly similar orientations to indicate the close similarity which extends beyond the first coordination sphere to lysine and arginine residues at the lower right hand side. The calcium ligands are:

Ca1 D144 O_δ^1, O_δ^2; L100 O; G102 O; G104 O; SOL478; SOL481
Ca2 D303 O_δ^1, O_δ^2; M259 O; G261 O; G263 O; SOL483
Ca3 E72 O_ϵ^1, O_ϵ^2; M28 O; G30 O; G32 O; SOL403

At the underside of the calcium binding loops solvent molecules are located bound to the carbonyl oxygens of K101, A103 (Ca1) and K260, A262 (Ca2). A solvent is also at the Ca3 loop, although less wel defined.

The structural features of the Ca1-3 binding sites are dissimilar to 'E-F hand' calcium sites of parvalbumins [18-20], but related to the calcium site of phospholipase A2 ([21], see Fig. 5) where the calcium is also sequestered by a glycine-rich loop and a spatially close but sequentially discontinuous Asp residue [21].

Calcium prefers seven-coordination by oxygens located approximately at the vertices of a pentagonal bipyramid [19]. The three calcium ions in annexin V have 6 oxygen ligands and are unsaturated by coordination and charge therefore. The seventh unoccupied coordination site may accept the phosphoryl moiety of

[a] The acronymes NATI, LAGP, CAPL, EDLA define crystal species and are explained in Table I;
EDTA = disodium ethylene dinitrilo tetraacetic acid

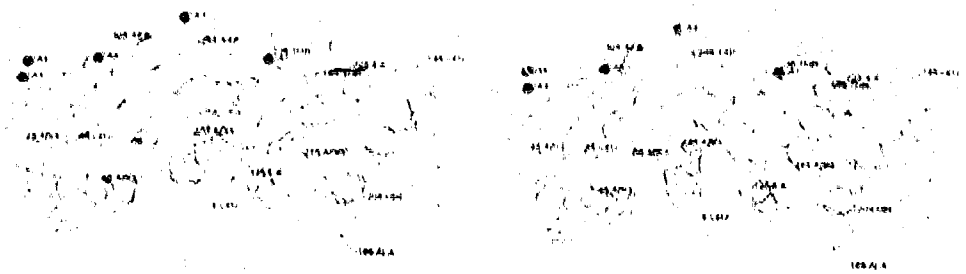


Fig. 2. The five calcium sites Ca1–Ca5 superimposed in the C_α-chain tracing. Ca1 is in turn IIA–IIB, Ca2 in IVA–IVB, Ca3 in IA–IB, Ca4 and CA5 are the main lanthanum sites and bound to repeat 1.

an acidic phospholipid. The conserved Lys and Arg residues and the amido N–H groups of the first glycines might stabilize this interaction (Ca1: K97, K101, G102; Ca2: K260, G261; Ca3: R25, K29, G30). A sulfate ion has been identified at this site coordinated to Ca3, R25 and R62 supporting this model.

The two sites occupied by lanthanum are coordinated very differently to three proteinaceous oxygen ligands:

Ca4: E35 $O_\epsilon{}^1$, $O_\epsilon{}^2$; T33 O; E36 (this side chain is not well ordered); and several waters
Ca5: E78 $O_\epsilon{}^1$, $O_\epsilon{}^2$; L73 O and waters

The calcium and lanthanum binding experiments have been conducted to obtain a measure of their relative binding strengths. The LAGP-CAPL difference map shows high peaks at sites Ca4(33σ), Ca5(17σ), Ca1(5σ), Ca2(5σ).

The difference map of EDLA-CAPL is very similar to LAGP-CAPL but shows roughly proportional lower occupations. In EDLA the crystals had to be soaked in 10 mM EDTA solution to remove bound calcium. But this had no effect on the three main calcium sites. The lower occupation is probably due to the lower lanthanum concentration in EDLA. In LAGP and EDLA low occupation is observed at Ca1 and Ca2 but not at Ca3.

Ca1–Ca3 form a distinct class of strongly bound caleiums by their common structural features (Fig. 4), their resistance towards EDTA (under the given experimental conditions), and their very slow exchange with lanthanum. Exchange by lanthanum follows the order: Ca1 > Ca2 > Ca3. Ca4 and -5 are highly occupied lanthanum sites. They show electron density peaks also in CAPL indicating loosely bound calcium.

Annexin V displays its full phospholipid binding capacity below 1 mM Ca^{2+} at physiological ionic strength [22], much lower than used in CAPL. The Ca1–3 binding loops, however, have average temperature factors of 39.7 Å^2 which are substantially higher than the total average value of 24.3 Å^2, and indicate enhanced mobility and disorder, possibly a consequence of the high ionic strength at which crystallization occurs. Polar interactions are weakened under these conditions.

3.3. *Calcium sites in other annexins*

We regard the -G-(A,L,M)-G-T-(39 residues)-(D,E)

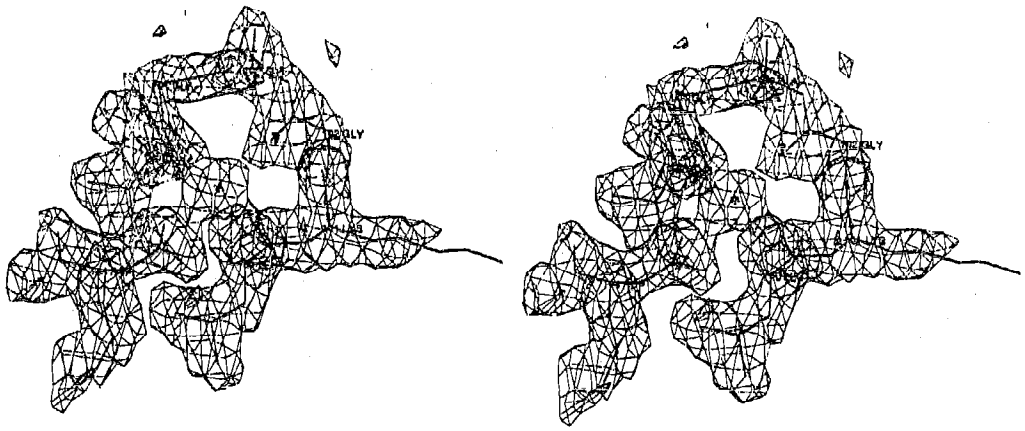
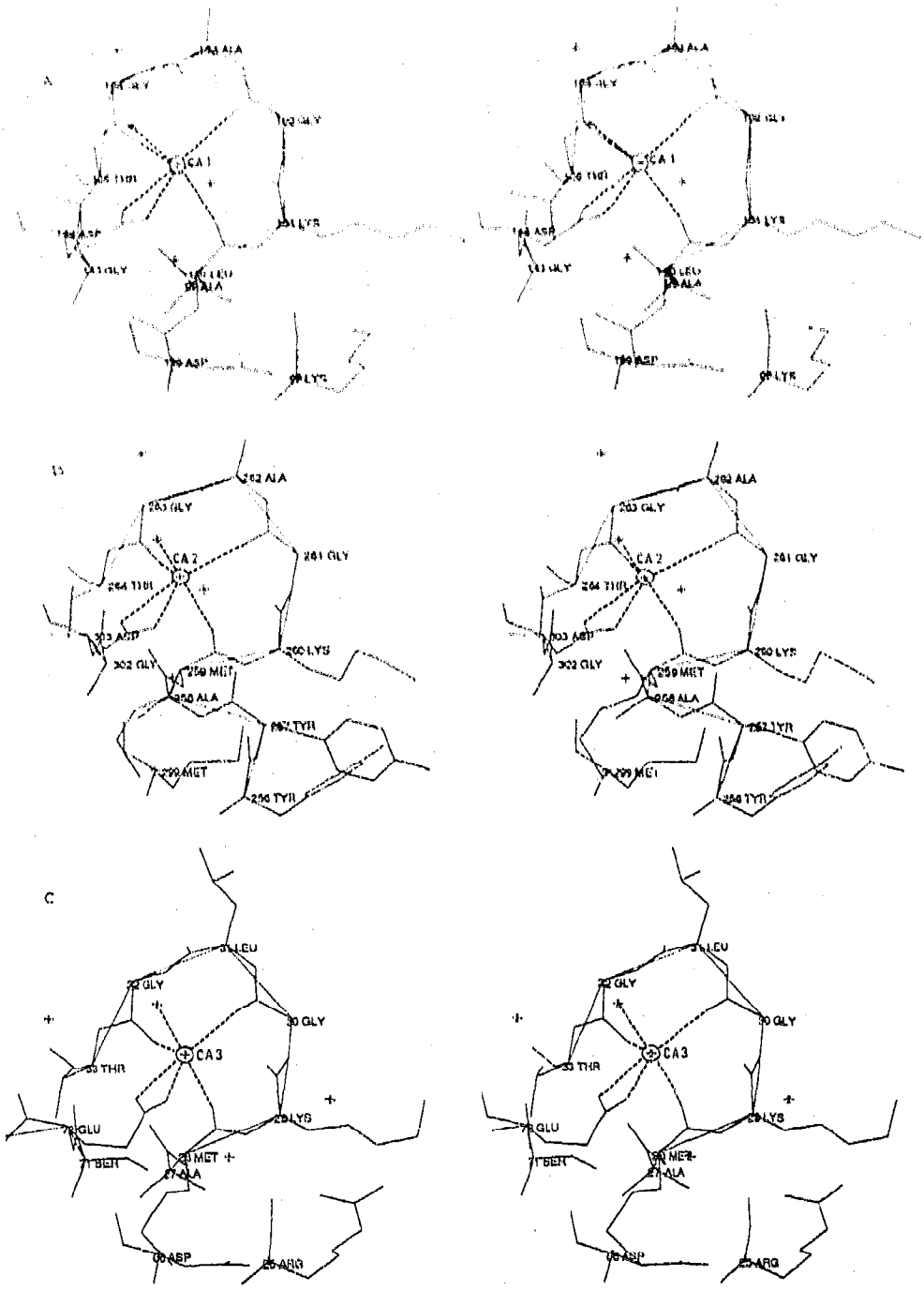


Fig. 3. The model and associated electron density of Ca1 and its ligands.



sequence as diagnostic for prototypical annexin calcium sites. It is found in all amino acid sequences[b] in repeats II and IV, often in repeat I like in annexin V, but not in the lipocortins I and II (calpactin I, II, annexin I, II, see [23] and never in repeat III. Analogously annexin VI (p68) with eight repeats has this sequence in repeats I, II, IV, V, VI, VIII. The invariant calcium sites 1 and 2 are symmetrical at the upper wide opening of the funnel-shaped central pore through the molecule (Fig. 5).

There are few data of the stoichiometry of calcium binding to annexins due to complications by the synergism with phospholipid binding and the relatively low affinity. For lipocortin I (annexin I) 3–4 mol calcium per mol protein have been found [36] in approximate agreement with our results.

[b] Amino acid sequences were taken from MIPSX sequence library (Martinsried). Original references cited in Huber et al. (1990)

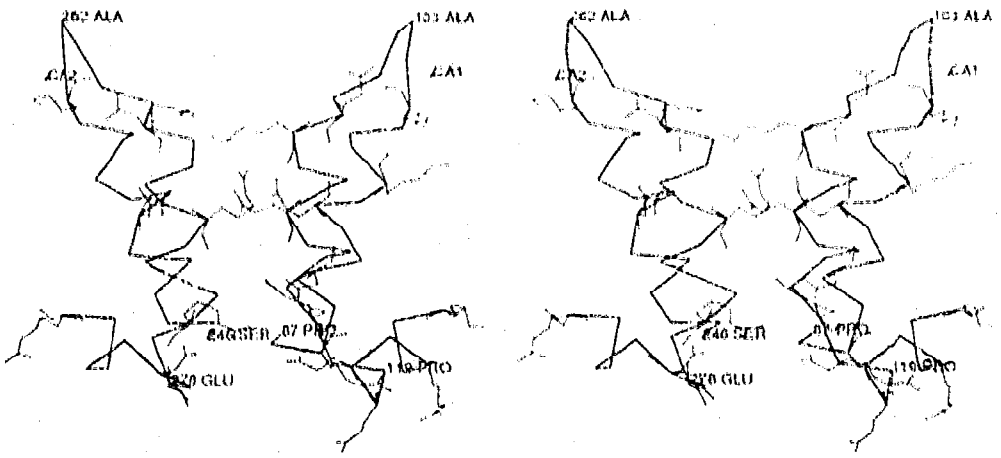


Fig. 5. Helices IIA, IIB and IVA, IVB are the framework of the central pore. It is lined with charged residues which are drawn.

3.4. *Implications for membrane binding*

The calcium binding sites identified so far are at the convex face of the molecule which had already been proposed as the probable membrane attachment site [1] based on p11 binding to p36 (annexin II). Protein p11 binds to the N-terminal tail of p36 located at the concave face [24–27].

Binding of anionic phospholipids, to annexin V is calcium dependent [22,35]. The phospholipid calcium interaction might be direct or allosteric. The observation that calcium binding in CAPL causes very little structural change at the calcium sites compared to NATI and the hexagonal form structure argues for the former. The unoccupied seventh coordination site in Ca1–3 and its occupation by a sulfate at Ca3 suggests that the phosphoryl moiety is in direct contact with the calcium. Additional interactions of the acidic head groups with the conserved basic residues mentioned might strengthen binding. There is no nearby hydrophobic binding site for the bis-acyl moiety of a phospholipid.

The calcium and putative phospholipid-binding sites are at protruding loops and constitute the probable docking sites of annexin to membranes. Annexin VI (p68) monolayer interaction causes an increase in surface pressure indicating at least partial insertion [28] and/or generation of disorder and expansion of the covered membrane area. The peripheral wall of the annexin molecule is polar (see Fig. 5b in [1]). Deep penetration is improbable therefore, except when accompanied by an extensive rearrangement of the surrounding phospholipids ('inverted micelle'). Alternatively there may be mainly surface attachment through the specific calcium phospholipid headgroup interaction associated with local restructuring of the proximal and, to a lesser degree, the distal leaflet of the membrane. Local rearrangement of the phospholipid under the docking area may make the membrane permeable, specifically between Ca1 and Ca2 which border the central polar pore through the molecule. In this hypothetical protein membrane complex the central local axis of symmetry which marks the pore is perpendicular to the membrane plane. We had associated this pore with the calcium specific voltage-gated channel of annexin VII (synexin) and V (endonexin II) on structural grounds [1] (see [6,9,29] concerning channel properties). The putative channel is shown in Fig. 5. It is formed by the symmetry-related helix IIA–Ca1 turn–helix IIB and helix IVA–Ca2 turn–helix IVB motifs, respectively. The channel is funnel-shaped with the open end at the calcium ions. 10 charged, 6 acidic and 4 basic residues, of which most are invariant in the annexin family, project into the channel and fill it together with a few bound solvent molecules. These latter indicate permeability of the channel.

Voltage-gating is a particularly interesting property of the annexin channel. There are different ways by which an electric field may act on the channel. Charged residues within the channel may rearrange or a global relative motion of the modules may occur. Calculations of the electrostatic potential have shown that the annexin V molecule is strongly dipolar. The arrow superimposed on the C_α-plot of annexin V (Fig. 1) connects the centre points of the positive and negative electrostatic potential calculated from the atomic coordinates for pH 8.5. It documents the substantial asymmetry in the charge distribution between modules (I,IV) and (II,III). An electrical field across the membrane would exert different forces on the modules and may cause a relative motion of the kind seen in Fig. 1. Rearrangement of individual residues or the modules as a whole may stabilize the open or closed states of the central channel and thus regulate calcium inflow.

3.5. *Other calcium, phospholipid-binding proteins*

Phospholipase A_2 requires calcium for enzymatic activity and hydrolyses aggregated substrates several orders of magnitude faster than monomeric phospho-

lipids. A direct interaction between calcium and the phosphoryl moiety has been proposed in the productive enzyme substrate complex [21]. On the basis of the structural similarity of the calcium binding sites in annexin and phospholipase we propose a membrane anchor role also in the latter. Cobra venom phospholipase A_2 aggregates on membrane surfaces [30].

Protein kinase C also shows calcium-dependent membrane binding and binds at least 8 mol calcium per mol protein [31]. There is no convincing sequence homology with EF-hand calcium sites [32,33], and it also lacks annexin-like calcium-binding segments. There are obviously different ways by which calcium-dependent phospholipid-binding is achieved depending on other functional requirements of the proteins.

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