The High-Resolution Crystal Structure of Human Annexin III Shows Subtle Differences with Annexin V[†,‡]

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ABSTRACT: The structure of recombinant human annexin III was solved to 1.8 Å resolution. Though homologous to annexin I and V, the annexin III structure shows significant differences. The tryptophan in the calcium loop of the third domain is exposed to the solvent, as in the structure of annexin V crystallized in high calcium concentrations, although the annexin III crystals were prepared at low calcium concentrations. The position of domain III relative to the other domains is different from both annexin V and I, suggesting further flexibility of the molecule. The entire N-terminus of the protein is well-defined in the present structure. The side chain of tryptophan 5 interacts with the hinge region of the hydrophillic channel, which could have an effect on the potential mobility of this region, as well as on its possible calcium channel behavior.

Annexins form a distinct family of calcium-binding proteins, since their calcium-binding loop is different from that of the EF-hand family of proteins (Raynal & Pollard, 1994; Swairjo & Seaton, 1994). They are widely distributed in different species, tissues, and cell types, with about 13 members having been identified and characterized so far. Besides their common property of binding phospholipids in a calcium-dependent manner, the proteins of this family share a high sequence homology (about 80%). They are formed by a 4- or 8-fold repeat of 70 amino acid domains that are highly conserved and by a more variable N-terminal segment. It is generally assumed that this variable N-terminus may confer different properties on the different annexins. Solution studies have shown that there is one principal calcium binding site per domain (Raynal & Pollard, 1994).

The physiological role of annexins remains unclear, although several functions have been proposed, such as initiation of membrane fusion in exocytosis and endocytosis, inhibition of phospholipase A_2, inhibition of coagulation, and calcium channel activity (Raynal & Pollard, 1994). The fundamental property of all annexin cores, the calcium-dependent membrane binding, could be the underlying cause of all these functions.

Annexin III, also named lipocortin III or placental anticoagulant protein III (PAP III), has an apparent molecular mass of 33 kDa with 322 amino acids. On its 16 residue long N-terminus, annexin III carries a PKC[1] phosphorylation site (Stoehr *et al*., 1990). Like other members of the family, it has anticoagulant and anti-phospholipase A_2 properties *in vitro*.

While annexin V is fairly ubiquitous, annexin III is expressed almost exclusively in differentiated cells of the myeloid cell lineage (Coméra *et al*., 1989). Moreover, its expression increases during the differentiation of these cells into neutrophils or macrophages, where it represents 1% of the cytosolic proteins (LeCabec *et al*., 1992). *In vitro* it promotes aggregation of isolated specific granules in the presence of 1 mM calcium. In neutrophils and monocytes it is associated with cytoplasmic granules and translocates to the membrane in activated cells (LeCabec & Maridonneau-Parini, 1994). Finally, annexin III is the only annexin for which an enzymatic activity has been suggested, namely, that of inositol 1,2-cyclic phosphate 2-phosphohydrolase (Ross *et al*., 1990).

The crystal structures of annexin V (Huber *et al*., 1992) and of the conserved core of annexin I (Weng *et al*., 1993) have been solved to date. These structures show that a sequence domain corresponds to a structural one, consisting of five α-helices (A−E). The four domains are arranged in a cyclic array, giving the molecule an overall flat, slightly curved shape with a convex and a concave face. The calcium binding loops are located in each domain between helices A and B on the convex, membrane binding face of the protein, while the N- and C-termini lie on the concave face (Huber *et al*., 1992). The molecule is highly symmetrical, with domains I + IV and domains II + III forming tight modules that are related by a pseudo 2-fold axis, while further approximate 2-fold axes relate the individual domains within each module. Between the two modules lies a hydrophilic channel that is considered to be the locus of calcium channel activity observed *in vitro* for some annexins (Huber *et al*., 1992; Raynal & Pollard, 1994).

The conformation of the conserved core of annexins I and V is very close. The principal differences concern the calcium binding sites: while the A−B loops are conserved, the absence of an essential acidic residue in the D−E loop of domain I in annexin I (it is an alanine in the sequence) means that a calcium ion cannot be coordinated in this site. On the other hand, the A−B loop of domain III containing a lysine at its summit forms a calcium site in annexin I. In

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[‡] The coordinates of annexin III have been deposited with the Brookhaven Protein Data Bank and assigned the code 1AXN.

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[1] Abbreviations: PKC, protein kinase C; Trp, tryptophan; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; AS, ammonium sulfate; F_o and F_c, observed and calculated structure factors, respectively.

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most crystal forms of annexin V the calcium in domain III is absent and the A−B loop that contains a Trp at its summit is buried within the molecule. In the presence of high calcium concentrations, as well as in the presence of phospholipids, this loop undergoes a conformational change and becomes a calcium binding loop (Meers & Mealy, 1993; Sopkova *et al*., 1993, 1994). Spectroscopic studies have shown that the Trp side chain makes a contact with specific phospholipids in the membrane, at the level of the ester carboxyl oxygen and the first groups of the acyl chains (Meers & Mealy, 1994).

In order to understand the differences in the function of annexin III compared to the other known annexins, we have solved its three-dimensional structure by X-ray crystallography.

MATERIALS AND METHODS

Annexin III cDNA was obtained from a PUC plasmid (BIOGEN, Cambridge, MA) using the *Sec*I restriction enzyme. Oligonucleotides were linked to complete the cDNA sequence encoding annexin III and to create a cloning site. The cDNA sequence was then cut by *Bam*HI and *Eco*RI restriction enzymes and introduced into a PGEX 2T vector (Pharmacia, Paris, France) to produce a GST fusion protein. One colony of *Escherichia coli* NB42 strain transformed with this plasmid was introduced into 3 mL of LB medium containing 50 mM ampicillin, grown at 37 °C overnight, and then introduced into 3 L of LB medium (containing 50 mM ampicillin). The growth was continued for about 2 h until the optical density was 0.6 at 600 nm, when IPTG (0.15 mM) was added. The protein was then expressed overnight at 25 °C. The bacteria were lysed, and the soluble fraction was purified on a column based on the affinity of GST for reduced glutathione. Thrombin cleavage from the GST moiety (1000 units, 1 h, room temperature with stirring) gave a full-length annexin III extended at its N-terminus by a glycine and a serine residue derived from the thrombin-sensitive peptide. This protein was applied onto a mono-Q FPLC column (Pharmacia) in 20 mM ethanolamine, pH 8.2, and eluted with a NaCl gradient. Each peak was analyzed by SDS−PAGE using 12% polyacrylamide. The purity was tested by silver staining and on an isoelectric focusing gel. The protein was concentrated on a YM10 (Amicon) membrane under nitrogen pressure and through a Centricon filter to a final concentration of 36 mg/mL.

Tryptophan fluorescence emission spectra were recorded between 300 and 420 nm on a SLM 8000 spectrofluorometer (Urbana, IL) with an excitation wavelength of 295 nm (2 nm bandwidth). Buffer blank (50 mM Tris-HCl at pH = 7.5) was subtracted under the same experimental conditions. Rayleigh and Raman scattering was minimized by crossed Glan-Thompson polarizers. The protein concentration was 0.3 mg/mL.

Parallelepipedic crystals were obtained by vapor diffusion using ammonium sulfate (AS) as a precipitant. The drop of 2 μL contained 18 mg/mL protein, 10 mM $CaCl_2$, and 25% AS in 50 mM Tris-HCl buffer at pH 7.5. The well contained 50% AS in the same buffer.

The crystals are monoclinic, space group $P2_1$, with cell dimensions of $a = 42.55$ Å, $b = 69.8$ Å, $c = 50.95$ Å, $\beta = 95.55°$, and one molecule per asymmetric unit.

Data were measured on the DW32 station on the DCI synchrotron ring at LURE, Orsay, which is equipped with a MaResearch imaging plate detector (Fourme *et al*., 1992), using a wavelengh of 0.9 Å and a crystal-to-detector distance of 160 mm. The data reduction was performed using the MarXDS program (Kabsch, 1993) and the CCP4 program suite (CCP4, 1979). The data quality is summarized in Table 1.

Table 1: Diffraction Data Statistics

resolution (Å)	23.80−1.78
total no. of observations	88603
no. of reflections	25718
R_{sym}[a]	0.043
completeness of data	92.0%
completeness in the last bin (1.83−1.78 Å)	79.2%
$I > 2\sigma(I)$	87.6%

[a] $R_{sym} = \sum(I_i - \langle I \rangle)/\sum I_i$ for equivalent reflections, where I_i is the intensity of an individual measurement and $\langle I \rangle$ is the mean value.

The structure was solved by molecular replacement using the structure of annexin V (Lewit-Bentley *et al*., 1994) as a search model, with the program AMoRe (Navazza, 1994). The correct solution was given by the highest peak in the rotation function (height 9.1 with the next peak at 5.4, data to 3.5 Å), as well as in the translation function (calculated at 2.5 Å resolution). After the final "fitting" stage (at 2.0 Å resolution) the final correlation factor was 57.4%, corresponding to an *R*-factor of 45.0%.

Refinement was initiated with X-PLOR (Brünger *et al*., 1987), using the rigid body option with each domain divided into five helices and five loops, followed by 100 cycles of conjugate gradient minimization. At this point, side chains of annexin V were changed to correspond to the annexin III sequence. A $3F_o - 2F_c$ map was calculated and almost all the side chains of the first, second, and fourth domains were fitted into the electron density map. In the third domain they were kept as alanines and the domain was refined as rigid bodies consisting of five helices and five loops based on a secondary structure prediction of annexin III (Rost & Sander, 1994; Rost *et al*., 1994), keeping the rest of the structure fixed. While our initial strategy of using the helices given by the known crystal structure of annexin V did not lead to significant movements and did not give a model with enough information in the electron density map for rebuilding the third domain, the rigid body refinement based on helices predicted by the PHD program (Rost *et al*., 1994), with an accuracy superior to 82% for the helix conformation, led to important movement of most helices and loops of the third domain (Table 2). This model was good enough to start the rebuilding of the entire domain using manual reconstruction on a graphics terminal with FRODO (Jones, 1978). With hindsight, this is understandable, since the conformation of the third domain corresponds to the "high-calcium" annexin V structure (Sopkova *et al*., 1993) rather than to the model used. The difference between the two structures is up to 13 Å for the residues in the A−B and D−E loops in this domain. After a conjugate gradient minimization and *B*-factor refinement, the *R*-factor was 27.7%, while it was 40.5% after the rigid body refinement. The N-terminal part of the protein from residues 3 to 10 was reconstructed using an "omit" map (Brünger *et al*., 1987). Further rounds of refinement consisting of simulated annealing were finally completed with PROLSQ (Konnert & Hendrickson, 1980) to obtain good geometry. Table 3 summarizes the refinement results.

Table 2: Division of Domain III into Units for Rigid Body Refinement[a]

	prediction for annexin III	movement (Eulerian angle/ translation in Å)	model structure	movement (Eulerian angle/ translation in Å)
loop domain II−III	160−172	0.0 2.1 −1.9/0.0 −0.2 0.3	160−172	−0.8 0.0 −0.2/0.0 −0.1 0.1
helix A	173−185	−3.8 3.7 2.7/0.0 −0.3−0.4	173−188	−0.6 0.9 0.4/−0.4 0.0 −0.4
loop A−B	186−195	−5.7 −3.5 6.3/−0.7 0.1 −1.3	189−194	−0.8 −1.1 2.0/−0.1 −0.2 0.1
helix B	196−200	1.7 6.7 6.3/−0.1 −0.2 0.7	195−204	0.5 3.2 2.2/−0.4 0.1 1.0
loop B−C	201−206	−4.3 −1.2 0.0/−0.1 −0.3 1.2	205−206	0.0 0.9 0.0/−0.9 0.3 1.4
helix C	207−219	10.1 1.5 7.5/1.2 0.4 0.5	207−221	3.6 1.1 −2.9/−0.7 −0.4 1.8
loop C−D	220−223	5.2 10.4 1.6/2.4 0.9 −0.3	222−224	−1.1 −2.3 0.6/−0.4 −0.8 1.5
helix D	224−232	−4.8 5.7 3.5/0.8 0.9 −0.8	225−228	−0.1 1.7 1.8/0.2 −0.4 0.9
loop D−E	233−236	−1.6 0.2 9.2/−0.3 0.2 −1.1	229−235	−1.2 −0.7 0.6/0.3 −0.9 0.4
helix E	237−247	0.2 1.7 −0.5/0.0 0.0 0. 0	236−249	−0.2 0.7 −1.7/0.0 0.0 0.0

[a] The sequence numbering is based on our convention for annexin III (see text).

Table 3: Refinement Statistics

total no. of non-hydrogen atoms	2557
no. of solvent molecules	283
no. of calcium ions	5
amplitude cutoff	$2\sigma(F)$
no. of reflections used	25485
R-factor[a]	0.173
R-free[b]	0.242
mean *B*-factor (Å^2)	20.2
rms[c] deviations from standard values	
bonds (Å)	0.009
planes (Å)	0.006
chiral volumes (Å^3)	0.088
B-factor on main-chain bonds (Å^2)	1.201
Ramachandran plot	
residues in most favored regions	94.9%
residues in further allowed regions	5.1%

[a] *R*-factor $= \sum(||F_o| - |F_c|)/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. [b] *R*-free defined in Brünger (1992). [c] rms, root mean square.

RESULTS AND DISCUSSION

The overall structure of annexin III resembles that of the other known annexin structures, as expected (Figure 1). The amino acid sequence is close to that of annexin V, especially as far as domain III is concerned. (The sequence is 49.5% identical between entire annexins III and V and 45.9% identical in domain III.) It was a surprise, therefore, to find that the structure in this domain corresponds more to that of annexin I. The A−B loop lies on the surface of the molecule, and a high-affinity calcium site is formed there, thus bringing the Trp side chain onto the surface of the molecule. Such a conformation is induced by high calcium concentrations in annexin V (Meers & Mealy, 1993; Sopkova *et al*., 1993, 1994) and at the concentrations used in our preparations has never been observed.

We therefore checked the behavior of the third domain in solution using fluorescence spectroscopy and titrating the annexin III by calcium. Annexin III contains two Trp residues, the second one lying at the N-terminus and therefore most likely exposed to solvent. The steady-state fluorescence emission of Trp190 and Trp5 in annexin III with no calcium is maximal at 341 nm (Figure 2), while in annexin V its maximum is at 325 nm for Trp187 under the same conditions (Sopkova *et al*., 1994) (we have numbered the annexin III sequence starting at its N-terminus as observed in the crystal structure, which makes Trp190 correspond to Trp187 in annexin V). The steady-state fluorescence emission spectrum of the tryptophans in annexin III at 0 mM Ca corresponds more to that of tryptophan in annexin V saturated with calcium. This suggests that the tryptophans in annexin III are much more exposed to the solvent than in annexin V even in the absence of calcium, confirming the conformation of the calcium loop of the third domain found in our crystal structure. Moreover, there is no significant perturbation of the tryptophan microenvironnement by calcium binding (Figure 2). We observed weak quenching at 1 mM Ca and a small red shift at 20 mM Ca which, however, cannot be compared with the large red shift observed in annexin V titrated by calcium (Sopkova *et al*., 1994). We therefore conclude that in annexin III the A−B loop in domain III is exposed (and accessible to calcium binding) even in the absence of calcium, unlike annexin V, and in spite of a very close sequence homology.

As far as the calcium sites in annexin III are concerned, their substitution corresponds most to the high-calcium form of annexin V (Sopkova *et al*., 1993). The principal site in domain III contains a highly occupied, highly ordered calcium ion (but no sulfate ion, contrary to the high-calcium form of annexin V). We find another calcium ion in domain I, but with a lower occupancy factor, an even less occupied site in domain II, where the liganding loop is becoming disordered, and no calcium in domain IV. In annexin V it was found (Sopkova *et al*., 1993) that, upon increasing the calcium concentration during crystal preparation, secondary calcium sites appeared in domains III and I, but no calcium was bound in domains II and IV. In our case, no further Ca sites are visible in domain I, while two types of secondary sites in domain III are partially occupied even at the relatively low calcium concentrations used. These sites are analogous to sites Ca4 and Ca5 described by Huber *et al*. (1992) for domain I in annexin V (Figure 3).

The packing in the annexin III crystals is totally different from that of any annexin V crystals prepared under similar crystallization conditions. If the presence or absence of calcium ions in domains II and IV could be attributed to different packing constraints (and thus accessibility to the sites), it is clear that the highly ordered calcium site in domain III is the most important site to investigate. Our structure could be confirming that it is this site that binds to the membrane *via* its calcium first, the exposed Trp side chain helping to anchor the molecule on the membrane surface. The less substituted calcium sites in the other domains would then fix onto the membrane after an adjustment of the molecule to the membrane surface.

In the annexin I and V structures, domains I + IV and II + III form modules that are related by a pseudo 2-fold axis (Huber *et al*., 1992). The relative position of the two modules is somewhat different in different crystal forms of annexin V (Lewit-Bentley *et al*., 1992), suggesting a possible motion about a hinge at the concave side of the molecule. A

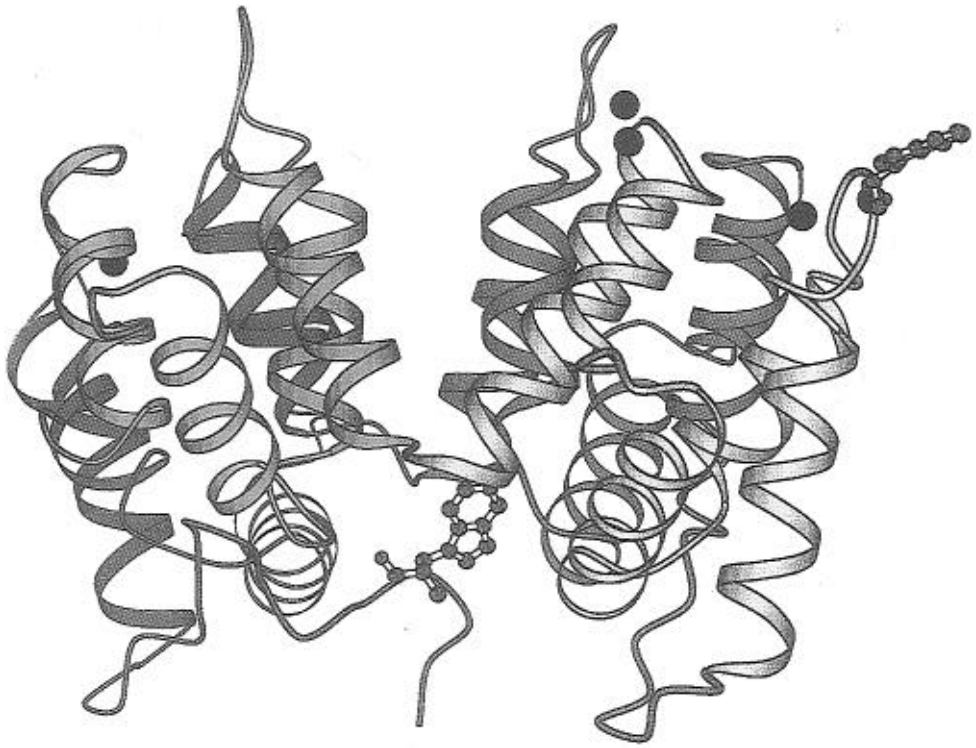

FIGURE 1: An overall view of the annexin III structure. Residues Trp5 and Trp190 and calcium ions (in black) are highlighted. Each of the four domains corresponds to a different color: domain I, dark blue; domain II, red; domain III, yellow; and domain IV, cyan. Module I containing domains I and IV is on the right and module II composed of domains II and III on the left of the figure.

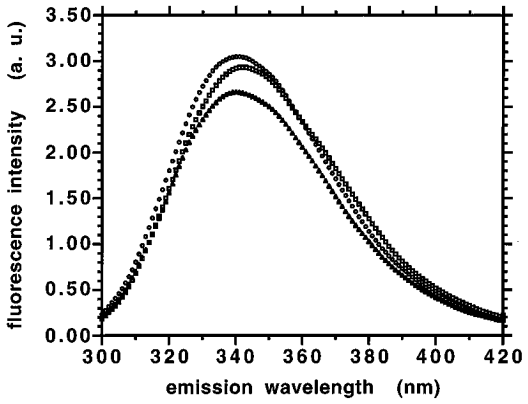

FIGURE 2: Fluorescence emission spectra of Trp5 and Trp190 in annexin III as a function of calcium concentration: (●) calcium-free protein, (◆) protein with 1 mM Ca, and (▲) protein with 20 mM Ca. Excitation wavelength = 295 nm. Protein concentration = 0.3 mg/mL.

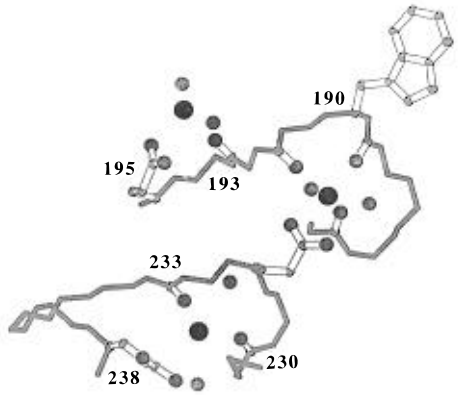

FIGURE 3: Calcium sites in domain III. The principal site in the A−B loop which contains Trp190 is on the right; the Ca4-type site coordinated by the carbonyl oxygen of 193, both oxygens of the Asp195 carboxyl group, and two water molecules is above. The Ca5-type site coordinated by the carbonyl oxygens of 230 and 233, Oϵ1 of Glu238, and two water molecules is below in the plane of the figure. Calcium atoms are the large black circles, while oxygens are smaller and dark gray.

comparison of the annexin III structure with those of annexin I and V shows a different relative position of domains. The central 2-fold axis is no longer conserved [a rotation of 166° is necessary to bring domain III into coincidence with domain I, while for annexins I and V this rotation is 178° and 179°, respectively (Figure 4)]. Yet the 2-fold axis is conserved as far as the superposition of domain IV on domain II is concerned (185°, 176°, and 179° for annexins III, I, and V, respectively). Furthermore, while the supplementary approximate 2-fold axis relating domains I and IV seems to be conserved in all three annexins, the symmetry between domains II and III has broken down in the case of annexin III, suggesting a relative movement of domain III in this crystal form. Annexin III may thus be showing us another point of flexibility of the molecule, given by the relative position of the four domains. The recent higher resolution electron microscopy studies of annexin V bound to phospholipid monolayers (Berendes *et al*., 1993; Voges *et al*., 1994; Olofsson *et al*., 1995) show an electron density that cannot be fitted by the crystallographically obtained annexin structure without applying a twist to the relative position of domains II and III. The situation in annexin III may be indicating such a change at atomic detail.

The annexin structures solved so far concerned essentially the conserved C-terminal region: annexin V has a rather short N-terminus (13 residues), while the annexin I structure concerned a protein with a cleaved N-terminus (Weng *et al*., 1993). We have crystallized an intact annexin III molecule, with its entire N-terminus of 16 amino acids plus an extra serine residue. Given the high order of the structure, the N-terminus is perfectly defined. It first runs along the

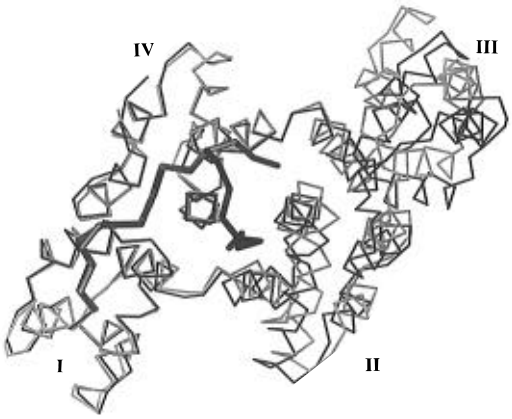

FIGURE 4: Superposition of the Cα backbone of annexin III (black) on annexin V (gray), viewed down the local 2-fold axis (this view is perpendicular to that in Figure 1). The N-termini are highlighted by thicker lines.

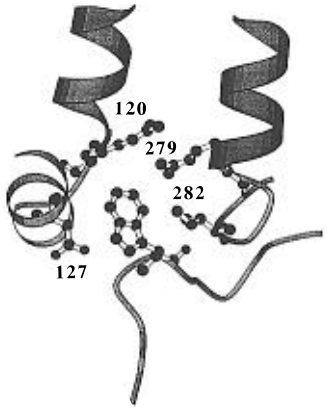

FIGURE 5: Insertion of the N-terminal Trp5 side chain in the hydrophilic channel in the center of the annexin III molecule. Trp5 forms a hydrogen bond with Glu127 and is in van der Waals contact with Ile282. Arg120 (domain II) and Arg279 (domain IV), which were implicated by Huber *et al*. (1992) in the calcium channel activity, are also shown. All figures (except Figure 2) were prepared using MOLSCRIPT (Kraulis, 1991).

concave face of the molecule under domains I and IV (rather like in the annexin V structures), but instead of remaining close to domain IV it continues toward the junction between modules of domains I + IV and II + III (Figure 4). At residue Trp5 (this Trp occurs only in the annexin III sequence) the N-terminus turns away from the surface of the molecule toward the solvent (which would be the cytosol in a cell). The side chain of Trp5 points into the molecule and lies, in fact, in the hydrophilic channel between modules that was suggested as the calcium channel (Huber *et al*., 1992). It makes hydrogen bonds and van der Waals interactions with several amino acids from domains I, II, and IV (Figure 5). In this position it may well be locking the hinge region of the structure and could thus modify this annexin's behavior. We suggest that both the flexibility and the accessibility of this hinge region could be altered and, as a consequence, membrane binding and calcium channel activities seriously affected.

As far as the putative enzymatic activity of annexin III is concerned, it has been suggested (Huber *et al*., 1992) that the secondary calcium sites in domain I could bind magnesium and thus be involved in the active center. These sites are not occupied in our structure at all, while the equivalent sites in domain III would not be easily accessible when the protein is bound to the membrane. On the other hand, the N-terminus with its specific sequence is accessible to the solvent and seems more likely as a potential active site. Nevertheless, the conformation of the N-terminal side chains in the crystal structure does not suggest an obvious site for phosphohydrolase activity.

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