Hinge-bending motions in annexins: molecular dynamics and essential dynamics of apo-annexin V and of calcium bound annexin V and I

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Annexins are homologous proteins that bind to membranes in a calcium dependent manner, but for which precise physiological roles have yet to be defined. Most annexins are composed of a planar array of four homologous repeats, each containing five α-helices and associated into two modules. Annexin V forms a voltage-gated calcium channel in phospholipid bilayers. It has been proposed that the hydrophilic pore in the centre of the molecule may represent the ion conduction pathway and that a hinge movement in annexin V causes a variation of the inter-module angle and opens the calcium ion path. Here we present the results of molecular dynamics simulations of apo-annexin V and of calcium-bound annexin V and annexin I. The three simulations show significant differences in conformation and dynamics. The essential dynamics method was used to study the essential subspace of annexin V and showed that one of the essential motions corresponds to the postulated hinge motion. The hinge residues were located between repeats but belong to helices rather than to the links between helices. Calcium binding to annexin V led to a limitation of this hinge motion with more open conformations being favoured.

Keywords: calcium channel/computer simulation/concerted motion/flexibility/internal motion

Introduction

Annexins are homologous proteins that bind to membranes in a calcium dependent manner, but for which precise physiological roles have yet to be defined (for reviews see Moss, 1992; Liemann and Lewit-Bentley, 1995). Suggested properties include mediation of membrane fusion, anti-inflammatory activity, anti-coagulant activity, interaction with the cytoskeleton, calcium channel formation, inhibition of phospholipase A2 and cell signalling.

The three-dimensional structure of human annexin V [AnxV] has been solved (Huber et al., 1992) and shows that annexins are composed of a planar array of four (I–IV) homologous and structurally conserved domains, hereafter called repeats, each containing five (A–E) α-helices (Figure 1). As an example of the nomenclature used here, the expression IIAB would refer to the loop between helix A and B of repeat II, whereas the expression IVA,B would refer to helix A and B of repeat IV. All four repeats contain a potential type II calcium binding site involving the AB and DE loops located at the convex surface (Huber et al., 1992; Weng et al., 1993). Calcium binding to secondary sites with weaker affinities (type III binding sites) is also possible. Different crystal forms with different amounts of calcium bound have been reported: hexagonal (Huber et al., 1990), rhombohedral (Huber et al., 1990), monoclinic (Lewit-Bentley et al., 1992) and triclinic (Sopkova et al., 1993). The crystal structures of rat (Concha et al., 1993; Swairjo et al., 1995), chicken annexin V (Bewley et al., 1993) and human annexin I [AnxI] (Weng et al., 1993) have also been described. Recently the crystal structures of annexin II (Burger et al., 1996), annexin III (Favier-Perron et al., 1996), annexin IV (Zanotti et al., 1998), annexin VI (Benz et al., 1996; Kawasaki et al., 1996) and annexin XII (Luecke et al., 1995) were also reported. We previously reported mutational studies and molecular modelling of AnxI aimed at improving our understanding of the relationship between calcium binding, structure and function of AnxI (Trave et al., 1991, 1994a,b; Cregut et al., 1994).

In the present paper we are interested in molecular dynamics of AnxI and AnxV and on the influence of calcium binding on the structure and dynamics of these molecules. The annexin structure is arranged into two modules (repeats I/IV and II/III) with approximate twofold symmetry and separated by a groove. It is well-known that hinge-bending motions in proteins are important phenomena for biological activity (Gerstein, 1994). In annexins, two hinge-bending motions have been suggested: (i) comparison of several AnxV crystal structures suggests that the α angle between the modules (Figure 2a) could be a function of calcium binding (Huber et al., 1990; Lewit-Bentley et al., 1992; Sopkova et al., 1993) and that opening of this angle might be related to the in vitro calcium channel property (Berendes et al., 1993; Burger et al., 1994); (ii) experimental studies of membrane-bound AnxV indicate that the membrane induces constraints on the annexin convex surface, resulting in a rotation of the α dihedral angle between modules (Voges et al., 1994) (Figure 1b). Molecular dynamics (MD) simulations would appear to be a useful complementary tool to these experimental studies since they have been shown to provide an atomic description of motions that is not attainable experimentally (Karplus and Petsko, 1990; van Gunsteren and Berendsen, 1990). Although the simulation times usually remain short compared with the relaxation time of many biologically interesting large scale motions, MD and the essential dynamics (ED) method can still provide valuable insights into the experimental results (de la Cruz et al., 1994; Gilson et al., 1994; Kumar et al., 1994; van Aalten et al., 1995; Arnold and Ornstein, 1997; Chillemi et al., 1997; Peters et al., 1997).

We have used the MD and ED methods to study hinge-bending motions of annexins I and V and to investigate possible conformational and dynamics differences between apo-annexin V and calcium bound annexin V and annexin I.
Materials and methods

Starting structures

The initial atomic coordinates for the simulations of AnxV with and without bound calcium ions were taken from the X-ray structures of the rhombohedral form [Huber et al., 1990; protein databank entry: 1avr (Bernstein et al., 1977)] and the hexagonal form (Huber et al., 1990; protein databank entry: 1avh), respectively. The rhombohedral form (316 residues) included five Ca$^{2+}$ ions and 202 X-ray crystallographic water molecules. The hexagonal form (318 residues) included 137 X-ray crystallographic water molecules. The initial atomic coordinates for the simulation of AnxI with bound calcium ions (314 residues) were kindly provided by X.Weng (Weng et al., 1993). This structure included six Ca$^{2+}$ ions and 377 X-ray crystallographic water molecules. It corresponds to a truncated protein in which the first 32 residues are absent due to proteolysis, resulting in a protein with approximately the same length than annexin V. The simulations included X-ray crystallographic water molecules and a surrounding 9 Å shell of TIP3P water molecules (Jorgensen et al., 1983). The three simulated models are referred to in this text as follows: AnxV, 1avh annexin V (no Ca$^{2+}$); AnxV-Ca, 1avr annexin V (six Ca$^{2+}$); AnxI-Ca, annexin I (six Ca$^{2+}$).

Molecular dynamics simulations

Energy minimizations and molecular dynamics simulations were carried out on an IBM SP2 using four processors. Molecular display was performed on Silicon Graphics Iris 4D30 and O2 workstations using the Insight II molecular graphics program (1995).

All the molecular mechanics and molecular dynamics calculations were conducted using the AMBER all atoms force field (Weiner et al., 1986) as implemented in the AMBER 4.1 software (Pearlman et al., 1995). An 8 Å residue-based cut-off distance for non-bonded interactions was used throughout. This means that if any atom of one residue is within 8 Å of any atom of another residue, every atom in each residue will see every atom of the other residue. Compared with atom-based cut-off, the average effective residue-based cut-off is thus increased by the average residue diameter. The following strategy was used to prepare each model (AnxV-Ca, AnxV, AnxI-Ca) for the MD simulation: the shell of water including X-ray crystallographic water molecules was first minimized until the r.m.s. deviation on the derivatives reached 0.1 kcal mol$^{-1}$ Å$^{-1}$, and then subjected to 10 ps of MD at 300 K with the protein and ions held rigid. After this equilibration stage, the protein–solvent system was minimized for several steps to a 0.05 kcal mol$^{-1}$ Å$^{-1}$ r.m.s. derivative. During the first step, constraints with a force constant of 100 kcal mol$^{-1}$ Å$^{-2}$ were applied to the backbone atoms (N, α-Carbon, C, O) and the Ca$^{2+}$ ions. Constraints with a force constant of 50 kcal mol$^{-1}$ Å$^{-2}$ were also applied to the side-chains of amino acids. Then, side-chain constraints were removed and backbone and ion constraints were gradually decreased from 100 to 5 kcal mol$^{-1}$ Å$^{-2}$. Finally, the last step of minimization was performed without any constraints.

For each model, a 1050 ps trajectory was calculated at constant temperature (300 K) after the system was heated gradually from 10 to 300 K during the first 15 ps. A time step of 1.5 fs was used and the non-bonded pair list was updated every 10 steps. Bond lengths were constrained to equilibrium values using the SHAKE algorithm (van Gunsteren and Berendsen, 1977).

Conventional analysis of the trajectories

Geometric analyses of the trajectories were performed with the CARNAL module of the AMBER 4.1 program (Pearlman et al., 1995). The root mean square (r.m.s.) deviations between conformations were computed, after best superimposition of the backbone of the two conformations, with the following equation:

$$\text{r.m.s. deviation} = \sqrt{\frac{\sum_{i=1}^{N} (x_{i}^1 - x_{i}^2)^2}{N}}$$

where $N$ is the number of atoms, and $x_{i}^1$ and $x_{i}^2$ are the coordinates of atom $i$ in conformations 1 and 2.

The r.m.s. fluctuation of an atom in an MD simulation was
obtained, after best superimposition of the backbone of all frames in the trajectory on the average structure, from the following equation:

$$\text{r.m.s. fluctuations} = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \langle x \rangle)^2}{N}}$$

(2)

where $n$ is the number of frames in the trajectory, $(x)$ the coordinate of the atom in frame $i$, and $\langle x \rangle$ the average coordinate of the atom in the trajectory. To compare these values with the experimental values, the $B$-factors in X-ray structures were converted to experimental fluctuations using the following equation (McCammon and Harvey, 1987):

$$B = \frac{8\pi^2}{3} \langle \Delta r^2 \rangle$$

(3)

where $\Delta r = x_i - \langle x \rangle$, and angle brackets denote time average.

The $\alpha$ and $\tau$ inter-module angles (Figure 2) were computed using the following strategy. The $\alpha$ angle was considered as the angle between two planes, each being defined by three points: plane 1 passes through the geometric centre of helices IIA,B,D,E, the geometric centre of helices IVA,B,D,E and the geometric centre of helices IC; plane 2 passes through the geometric centre of helices IIA,B,D,E, the geometric centre of helices IIC and IIV; the dihedral angle $\tau$ was defined as the angle between two planes: plane 1 passes through the geometric centres of repeats I, II and IV; plane 2 passes through the geometric centres of repeats II, III and IV. Loops AB and DE were excluded from calculation of the geometric centre of the repeats.

The putative ion channel dimensions were computed with the program HOLE (Smart et al., 1996). The same van der Waals radii were used as in a previous study of the pore dimensions of human annexin V (Smart et al., 1994). Six runs were performed to map the central pore.

Essential dynamics analysis

The essential dynamics method (Amadei et al., 1993) is based on the diagonalization of the covariance matrix (Ichiyi and Karplus, 1991).

$$C_{ij} = \langle (x_i - \langle x_i \rangle)(x_j - \langle x_j \rangle) \rangle$$

(4)

in which $x_i$ and $x_j$ are the separate $x$, $y$, $z$ coordinates of every atom and angle brackets indicate average over a trajectory from which overall rotational and translational motion have been removed. Diagonalizing the covariance matrix yields a set of eigenvectors indicating the directions of concerted motions of atoms and corresponding eigenvalues representing the mean square fluctuation of the total displacements along these eigenvectors (Amadei et al., 1993). The eigenvectors are usually ordered according to decreasing values of the eigenvalues, and it has been shown that the first few eigenvectors define an essential subspace in which most of the significant large-scale motions take place, whereas all other eigenvectors correspond to irrelevant local fluctuations. Only $\alpha$-atoms were used for the analysis since it has been shown that they are sufficient to give a correct picture of the essential dynamics (Amadei et al., 1993; Chillemi et al., 1997). The moving window superposition method (van Aalten et al., 1995) was used to search hinges in the AnxV structures. Computations were performed with the MATLAB program (1994) running on Hewlett-Packard 735 workstations.

**Results and discussion**

Conventional analysis of MD trajectories

For all simulations, after the initial thermalization (15 ps) the system was equilibrated for 235 ps and the subsequent 800 ps were used for analysis. To control the stability of the simulations, the potential energy, the root mean-square (r.m.s.) deviation from the starting structure and the radius of gyration were monitored. The potential energies did not vary significantly (data not shown). The r.m.s. deviations stabilize near 1.3 Å, a typical value for MD simulations performed in an explicit water environment (Brooks et al., 1988; Howard and Kollman, 1992; de la Cruz et al., 1994), and the radii of gyrations do not change appreciably during simulations (Figure 3). All these values indicate that no major conformational change occurred during the simulations.

AnxV calcium binding has been studied by small-angle neutron-scattering (Ravanat et al., 1992). These experiments showed that the radius of gyration of AnxV increases from 1.9 nm in the absence of calcium to 2.2 nm in the presence of 3 mM Ca$^{2+}$, suggesting significant shape modification. It has been supposed that this conformational modification arises through a hinge-bending motion of the type identified by comparison of different X-ray structures (Figure 2a). Indeed, calcium binding and temperature dependence of the CD spectra were found to be consistent with relative movement of rigid structural domains (Sopkova et al., 1994). However, the reported experimental $R_g$ variation due to the calcium binding (Ravanat et al., 1992) should correspond to a large conformational modification that was not observed in crystals with different calcium content. The range of available $\alpha$ inter-module angle deduced from the crystal structures was correctly reproduced in the AnxV and AnxV-Ca simulations but resulted...
in maximum and minimum observed radii of gyration of 2.16 and 2.11 nm (Figure 3B), close to the largest experimental value. It is a possibility that the protein undergoes much greater motions, and especially compaction motions, in solution than those deduced from the X-ray structures or seen in the MD simulations. It is worth noting here that all annexin crystals were grown in the presence of calcium, even the AnxV form with no bound calcium ions used in the AnxV simulation (Huber et al., 1990). Although the AnxV structure was stable in the simulation, it is conceivable that the solution conformation in the true absence of calcium displays a significant but unknown conformational change consistent with a smaller radius of gyration. It cannot be definitely excluded, however, that auto-association or solvation phenomena influenced the experimentally determined Rg.

The mean-square atomic positional fluctuations calculated from the simulations appeared consistently lower than the experimental fluctuations calculated from the crystallographic B-factors, although regions with highest simulated fluctuations roughly correspond to regions with highest B-factors (data not shown). The linear correlation coefficients between simulated and experimental fluctuations are 0.47 (AnxV), 0.38 (AnxV-Ca) and 0.50 (AnxI-Ca). Several explanations for discrepancies between experimental and simulated fluctuations have been proposed leading to the conclusion that these data should be compared very cautiously (Hunenberger et al., 1995). The mean-square positional fluctuations reported in Figure 4 show that, as expected, helical regions displayed lower fluctuations whereas loop regions (and especially the AB and DE loops) appeared more flexible. The fluctuations of these loops were slightly increased in the simulation of the calcium bound annexin V in comparison with apo-annexin V. This was rather surprising since the AB and DE loops are the main constituents of the calcium binding sites, and coordination to calcium would have been expected to reduce the accessible conformations for these segments. This appeared however not to be the case. Moreover, the DE loop of repeat III also displayed higher fluctuation in AnxV-Ca, although no calcium was bound in this repeat. AnxV repeat III has been shown to be able to bind calcium when calcium concentration is high (Concha et al., 1993; Sopkova et al., 1993), but this repeat adopted a different conformation in the crystal structures used here which did not allow calcium binding (Huber et al., 1992). The fact that the IIIDE loop still displayed larger fluctuations in the AnxV-Ca simulation is indicative of a certain degree of interdependence between calcium binding sites. Calcium binding to annexins is usually considered as non-cooperative, but cooperative calcium binding to annexin I has been observed for the K128E mutant (Trave et al., 1994a), indicating that some interdependence between calcium binding sites is possible. Beside the AB and DE loops, the segment joining repeats II and III displayed significant fluctuations in annexin V but not in annexin I.

Two kinds of motions involving modules (I/IV) and (II/III) of AnxV have been proposed. It has been postulated that these motions are related to two particular properties of the protein: (i) calcium channel activity (Demange et al., 1994) and (ii) membrane binding (Voges et al., 1994). The former motion corresponds to variation in the inter-module angle $\alpha$ (Figure 2A) and has been postulated from the comparison of crystal structures of annexin V (Huber et al., 1990; Lewit-Bentley et al., 1992; Sopkova et al., 1993), annexin I (Weng et al., 1993) and annexin III (Favier-Perron et al., 1996). It has been proposed that this flexibility ($\alpha$ angle) could influence the diameter of the central calcium channel between the two modules and consequently Ca$^{2+}$ permeability (Demange et al., 1994; Liemann et al., 1996). However it remained unclear to which extent the observed conformational change in the crystal structures was only a consequence of packing effects or a result of calcium load since the crystal forms were prepared under different conditions. The latter motion (tor dihedral, Figure 2B) was deduced from a structural study of two-dimensional crystals of AnxV bound to a phospholipid monolayer (Voges et al., 1994). In these crystals, membrane binding induced torsion between modules (Figure 2B, tor variation) rendering the calcium binding sites approximately coplanar and therefore providing better interaction with the phospholipid polar heads, as previously suggested (Huber et al., 1992). These two motions were analysed during the three MD simulations by calculating the $\alpha$ angle and the tor dihedral angles (see Materials and methods) every 0.3 ps (Figure 5).

Comparison of AnxV-Ca and AnxV simulations shows that, on average, the $\alpha$ angle between AnxV modules was larger in the simulation in which five calcium ions were bound than in the absence of calcium. The average values (30.3 and 25.3°, respectively) correspond to a difference of 5°, which is close to the maximal difference observed between crystal structures. Thus, the simulations strongly support the proposed hinge-

![Fig. 4. Total mean-square positional fluctuations in the Anx V (A), Anx V-Ca (B) and Anx I (C) simulations. The positions of the helices along the sequence are indicated by shaded rectangles: repeats I and III, black; repeats II and IV, grey.](image)
bending motion and suggest that the average inter-module angle is affected by the calcium load. If packing effects were prominent, then the simulations for AnxV-Ca and AnxV in solution should have converged towards an identical mean conformation. It could be argued however that the conformational sampling of the simulation was insufficient such that the conformations in each simulation remained close to the initial conformation. Although this cannot be definitely ruled out, it is worth noting that during both simulations the \( \alpha \) angle was able to temporarily explore values in the range of values sampled by the other simulation without converging toward it. Surprisingly, the \( \alpha \) angle in the Anxl-Ca simulation was closer to the angle in the AnxV simulation than to the angle in the AnxV-Ca simulation.

On the contrary, the average values of the \( \tau \) dihedral angle were only slightly different in the two annexin V simulations (mean values of 15.6 and 17.4 for AnxV and AnxV-Ca, respectively) and the variations remained small when compared with a postulated 34° variation when annexin binds to membrane (Voges et al., 1994). It must be stressed however that the simulations were performed in solution and in the absence of membrane. It is likely that larger motions take place at the water–membrane interface due to the external forces that the protein experiences when it approaches the membrane. Nevertheless, binding to membranes does not induce large conformational modifications (Voges et al., 1994). It is therefore postulated that the flexibility of annexins in solution may provide some clues about the possible domain motions that must take place at the water–membrane interface.

The Anxl-Ca simulation led to a decrease in the \( \alpha \) angle (from 27.5° at 0 ps to a mean value of 25.1°) and to an increase of the \( \tau \) dihedral angle (from 21.6° at 0 ps to a mean value of 25.5°). Both \( \alpha \) and \( \tau \) average values for Anxl-Ca are significantly different from those of AnxV-Ca. Explanations for these apparent discrepancies between Anxl-Ca and AnxV-Ca dynamics must await further experimental data on Anxl structure and dynamics. Still, it is possible that the differences mentioned above between annexin I and V might be related to the difference in the biological activities of these members of the annexin protein family. In any case, the large variations of the \( \alpha \) and \( \tau \) inter-module angles observed in the simulations demonstrate a clear flexibility of the annexin scaffold in accordance with the two hinge-bending motions previously postulated.

To locate flexible amino acids possibly involved in the above motions, r.m.s. fluctuations of the \( \varphi \) backbone dihedrals were plotted against the residue number (Figure 6). Plots of the \( \psi \) backbone dihedrals give very similar profiles (data not shown). Not surprisingly, the dihedral fluctuation of the residues included in the helices was much smaller than the fluctuation of residues in the loops. Examination of residues with \( \varphi \) fluctuations larger than 25° in the AnxV simulation showed that four out of eight are glycines (30, 102, 261, 306). The four other residues are located in the start of the loop IIAB (Ala103), the end of loop ICD (Ser136), the start and end of the link between repeat II and III (Asn160 and Ile167). It can be concluded from this observation that most of the loops underwent internal conformational changes mostly near glycine residues and otherwise displayed rigid-group motions allowed
by conformational changes at the start or at the end of the loop. Examination of AnxV crystal structures indicated that modules I/IV and II/III are linked by very short segments between helices IE and IIA (Lys86, Pro87) and between helices IIIE and IVA (Arg245, Ser246) which were supposed to be the hinge segments allowing the inter-module motions shown in Figure 2 (Huber et al., 1990; Huber et al., 1992). The dihedral fluctuations observed in the simulations indicate that beside the above residues, the first turn of helix IIA (residues 88–91) and of helix IVA (residues 247–249) also display some limited conformational flexibility suggesting that larger segment than those previously proposed are involved in the inter-modules motions.

As far as the residue flexibility is concerned, one particular remark can be done about histidines 98 and 267. An NMR study of annexin dynamics showed that these two residues located on opposite sides at the top of the putative calcium channel, display different flexibilities ($T_2$ relaxation times) in the absence of calcium although it was impossible to determine which of the two histidines was the most flexible (Neumann et al., 1994). In the AnxV simulation, we observed an approximate twofold increase of $\chi^i$ dihedral fluctuations between His98 and His267 (7.3 and 13.3°, respectively). This result is roughly consistent with the experimental mobility difference (Neumann et al., 1994), suggesting a possible selective assignment of the histidine signal with the lower $T_2$ relaxation time to His267. This is also in agreement with the observation that His267 was able to break its salt-bridge to Glu95 and to adopt a different solvent-exposed conformation in the crystal structure of the E112G annexin V mutant, thus identifying His267 as a weak ionic interacting partner (Liemann et al., 1996). Interestingly, this salt-bridge was also broken during the AnxV-Ca simulation, in which the intercalation of two water molecules between His267 and Glu95 occurred along with a 180° flip of the histidine ring.

**Essential dynamics analysis**

Figure 7 shows a plot of the eigenvalues against eigenvector indices, derived from the covariance matrices obtained from the individual AnxV, AnxV-Ca and AnxI-Ca trajectories. As already reported for other simulations, there are only few eigenvectors with large eigenvalues indicating that the protein motion in the essential subspace only occurs along very few directions (Amadei et al., 1993; van Aalten et al., 1995; Peters et al., 1997). The observation of similar eigenvalues (i.e. mean square fluctuation of total displacement) indicates that there is no significant change in the amplitude of the essential motions in the three simulations. As already discussed from the total mean square displacements (Figure 4), calcium binding does not seem to reduce the flexibility of the protein.

The motions within the essential subspace can be studied by projecting the trajectories onto individual eigenvectors. In Figure 8, the projections of the $Ca$ trajectories on the first three eigenvectors are shown. In the three simulations the motion 'period' is larger than the simulation time for the first eigenvector, approximately equal for the second eigenvector and significantly shorter for the third eigenvector. The components of the first three eigenvectors for the AnxV simulation are shown in Figure 9. It is apparent from Figure 9 that the motions in the essential subspace are not limited to loops, which display the largest total positional fluctuations (Figure 4A), but involve larger groups of atoms moving in a concerted manner. The residues that participate the most in the first eigenvector motion are located in the AB loops of repeats I and IV, in the link between repeats II and III and near the hinge region (residues 86 and helix IIA). With respect to the second eigenvector motion, the loops AB, CD, DE and helix D of repeat I as well as the AB loop of repeat II are mostly involved. The motion along the third eigenvector is distributed all along the sequence (Figure 9).

Perhaps the most interesting observation was the nice correlation between the displacement along the second eigenvector and the $\alpha$ inter-module angle (linear correlation coefficient –0.83). This correlation allows us to analyse more carefully the hinge-bending motion related to the $\alpha$ angle variations shown in Figure 2a. On the other hand no significant correlation could be found between the main eigenvector motions in any simulation and the $tor$ dihedral angle. A 3D representation of the motion along the second eigenvector is shown in Figure 10. The dotted line corresponds to the largest value along the eigenvector motion (i.e. a small $\alpha$ angle value, compare Figures 8B and 5A). In this motion, repeat I is clearly

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**Figure 7.** Plot of eigenvalues against corresponding eigenvector indices derived from the AnxV (solid line), the AnxV-Ca (dotted line) and the AnxI-Ca (dashed line) trajectories. The eigenvectors have been sorted by decreasing eigenvalue.

**Figure 8.** Projections of the simulation frames on the first three eigenvectors for AnxV (upper plots), AnxV-Ca (middle plots) and AnxI (lower plots).
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Methods (van Aalten et al., 1995). A window of 55 residues (approximately the length of four consecutive helices) was chosen and applied to the two extreme structures along the first three eigenvector motions. Figure 11 shows that there are basically three main hinge regions in the first two eigenvector motions, all being located near the junctions between repeats.

Hinge 1 in the second eigenvector motion, which is correlated with the $\alpha$-inter-module angle, is around residue Ala$^{81}$ in the middle of helix IIE and causes repeats I and II to move in a rocking-like behaviour with residues at the bottom moving away from the inter-module groove at the same time that residues at the top move toward the groove (Figure 10). Hinge 2 is around residue Leu$^{156}$ at the end of helix IIE. Therefore, and quite unexpectedly, helix IIE at the interface between repeats II and III also seems to be a hinge region involved in the essential motion correlated with inter-module angle variation. Hinge 3 is around residue Val$^{239}$ in helix IIIIE. The motion of repeat IV is similar to those of repeats I and II, although with a smaller amplitude. However the motion of repeat III is slightly different and the slight rocking-like motion is accompanied by a rotation around an axis running parallel and near helix IIIIE with a clockwise rotation associated with the opening of the groove (when looking from the convex surface, as in Figure 10). Interestingly enough, hinge 1 and 3 are not far from previously hypothesized hinge regions at the junction between repeats I and II on the one hand and repeats III and IV on the other hand (Huber et al., 1992; Kaneko et al., 1997). However, rather than non-helical residues Lys$^{86}$ and Arg$^{245}$, helices IE and IIIIE themselves seem to make the flexible hinges. Repeat III is bracketed by hinge 2 and hinge 3 and displays a specific rotation which is not observed for the other repeats. Interestingly enough, a similar rotation of repeat III has already been inferred from comparison of crystal structures of annexin III and annexin I and V (Favier-Perron et al., 1996). In annexin III, the groove is more closed than in annexin I and repeat III has rotated counter-clockwise when looking from the convex surface.

The annexin V ligand K-201 which inhibits calcium channel activity is supposed to act as an allosteric modulator by locking the hinge between repeats III and IV (Kaneko et al., 1997). The crystal structure of annexin V in complex with K-201 revealed that residues 5 (in the N-terminus), 118, 119, 161, 203, 207, 243, 244 and 247 lie in close proximity of the ligand (Kaneko et al., 1997). It is worth noting that residues 243 and 244 at the end of helix IIIIE and 247 at the start of helix IVA belong to hinge 3 and that residue 161 belongs to hinge 2 (Figure 11). Interestingly Arg$^{161}$ is one of the most conformationally flexible residues in the AnxV simulation whereas Ile$^{247}$ is the most flexible one in the third hinge region as shown by the $\phi$ fluctuations (Figure 6). These findings strongly support the possibility that calcium channel inhibition by K201 is, at least in part, a result of a restrained hinge motion due to binding of K201 near hinge 2 and hinge 3.

To evaluate the influence of calcium binding on the $\alpha$-angle variation during the dynamics, the AnxV-Ca and AnxI-Ca trajectories were projected onto the second eigenvector obtained from the AnxV simulation. To do this the average structures in the AnxV-Ca and AnxI-Ca simulations were first superimposed onto the average AnxV structure to remove overall rotation and translation between trajectories. The resulting plot (Figure 12) shows that the AnxV-Ca trajectory corresponds to lower values and display smaller amplitude along this eigenvector motion than does the AnxV trajectory. This result is remarkably consistent with the geometrical analysis of the $\alpha$-angle along trajectories (see above and Figure 5) and strongly reinforces the hypothesis that calcium binding to annexin V does influence the $\alpha$-inter-module angle and favours more open forms. The associated changes in channel dimensions were evaluated using the HOLE program (Smart et al., 1996). This program had previously been used to study the pore dimensions in the crystal structure of calcium-bound human annexin V (Huber et al., 1992) showing a channel constriction to 0.6 Å (Smart et al., 1994). As expected the channel was slightly enlarged (0.8 Å) in the most open form obtained in the AnxV-Ca simulation (largest $\alpha$ angle) and slightly reduced (0.5 Å) in the most closed form in the AnxV.
Fig. 10. 3D representation of the motion along the second eigenvector calculated from the AnxV Cα covariance matrix. The two extremes structures along this motion are shown. The upper plot shows AnxV viewed along the pseudo twofold axis. The N-terminus is behind helix IVA and the C-terminus behind helix IVC. All helices are labelled. The lower plot shows repeats I and II viewed perpendicular to the twofold axis. Loops AB and DE and helices C are labelled. The rocking-like motion is shown as arrows.

Fig. 11. Output from the moving window superposition method (window size 55 residues) using the extreme structures from the AnxV simulation projected onto the first (solid line), the second (dotted line) and the third (dashed line) eigenvectors.

Fig. 12. Projections of the simulation frames of the AnxV (solid line), AnxV-Ca (dotted line) and AnxI (dashed line) trajectories onto the second eigenvector calculated from the AnxV Cα covariance matrix.

Simulation. The largest channel constriction observed (0.8 Å) is still insufficient for the passage of ions suggesting that larger motions than those seen in the simulation would be necessary for ion channel activity.

Conclusion
Annexin V as well as other family members form voltage-gated calcium channels in phospholipid bilayers (Rojas et al., 1990; Voges et al., 1995; Liemann et al., 1996). It has been proposed that the hydrophilic pore in the centre of the molecule may represent the ion conduction pathway (Huber et al., 1992), and that a hinge movement in annexin V opens the calcium ion path (Huber et al., 1992; Kaneko et al., 1997). Molecular dynamics and the essential dynamics analysis of the trajectory of AnxV indeed showed such a hinge-bending motion in which each repeat is moving in a rocking-like fashion in and out
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from the inter-module groove. This motion involved three main hinges located near the junctions between repeats I–II, II–III and III–IV and comprising part of the adjacent helices IE, IIA, IIE, IIE and IVA. In this motion, repeat I is the most mobile part. As can be shown from the mean-square displacements (Figure 4), repeat I is also the most mobile part in the AnxI-Ca simulation. Although the modification of the size of the central channel remained limited in the hinge motion, the implication of helices IE, IIA, IIE, IIE and IVA, which delineate the channel, strongly supports the postulated involvement of this motion in the calcium channel property of annexin. We have shown that calcium binding does influence the conformation and dynamics of annexin V and tend to stabilize more open conformations, although the simulations did not display the large variations in the radius of gyration measured experimentally (Ravanat et al., 1992). The fact that the calcium concentration affects the conformation and dynamics of annexins is probably relevant to its biological function. It is likely that membrane binding also affects annexin conformation and dynamics and may amplify the hinge motion observed in the simulation in solution.

Clear differences were observed in the average conformations and in dynamics between AnxI and AnxV (i.e. the average values of the inter-module angles). It has been proposed that the N-terminus, which differs in length and in amino acid composition between annexin members whereas the core is strongly conserved, is the main determinant of activity differences between annexin members. The annexin V N-terminus is among the shortest ones and the first two residues were absent in the crystal structure. The N-terminus of annexin I is 27 residues longer but 32 residues were omitted in the simulations since they were not seen in the crystal structure used as starting conformation due to proteolyses. This resulted in simulated annexin I and annexin V with N-termini of approximately the same length. Nevertheless, although this should await further experimental support, we have observed differences between AnxI and AnxV in the simulations. Such differences might suggest that the protein core itself, through subtle modifications of its conformation and dynamics, could also carry part of the information to distinguish between annexins, complementary to the unique features provided by the specific N-termini.

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