Ca²⁺ and membrane binding to annexin 3 modulate the structure and dynamics of its N terminus and domain III

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Abstract

Annexin 3 (ANX A3) represents ~1% of the total protein of human neutrophils and promotes tight contact between membranes of isolated specific granules in vitro leading to their aggregation. Like for other annexins, the primary molecular events of the action of this protein is likely its binding to negatively charged phospholipid membranes in a Ca²⁺-dependent manner, via Ca²⁺-binding sites located on the convex side of the highly conserved core of the molecule. The conformation and dynamics of domain III can be affected by this process, as it was shown for other members of the family. The 20 amino-acid, N-terminal segment of the protein also could be affected and also might play a role in the modulation of its binding to the membranes. The structure and dynamics of these two regions were investigated by fluorescence of the two tryptophan residues of the protein (respectively, W190 in domain III and W5 in the N-terminal segment) in the wild type and in single-tryptophan mutants. By contrast to ANX A5, which shows a closed conformation and a buried W187 residue in the absence of Ca²⁺, domain III of ANX A3 exhibits an open conformation and a widely solvent-accessible W190 residue in the same conditions. This is in agreement with the three-dimensional structure of the ANX A3-E231A mutant lacking the bidentate Ca²⁺ ligand in domain III. Ca²⁺ in the millimolar concentration range provokes nevertheless a large mobility increase of the W190 residue, while interaction with the membranes reduces it slightly. In the N-terminal region, the W5 residue, inserted in the central pore of the protein, is weakly accessible to the solvent and less mobile than W190. Its amplitude of rotation increases upon binding of Ca²⁺ and returns to its original value when interacting with membranes. Ca²⁺ concentration for half binding of the W5A mutant to negatively charged membranes is ~0.5 mM while it increases to ~1 mM for the ANX A3 wild type and to ~3 mM for the W190 ANX A3 mutant. In addition to the expected perturbation of the W190 environment at the contact surface between the protein and the membrane bilayer, binding of the protein to Ca²⁺ and to membranes modulates the flexibility of the ANX A3 hinge region at the opposite of this interface and might affect its membrane permeabilizing properties.

Keywords: Annexin; tryptophan fluorescence; conformational change; protein-membrane-calcium interaction

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Abbreviations: ANX A3, annexin 3; ANX A5, annexin 5; W5A-ANX A3, annexin 3 mutant where the residue tryptophan 5 is replaced by ala-

nine; W190A-ANX A3, annexin 3 mutant where the residue tryptophan 190 is replaced by alanine; E231A-ANX A3, annexin 3 mutant where the residue glutamic acid 231 is replaced by alanine; D226K-ANX A5, annexin 5 mutant where the residue aspartic acid 226 is replaced by lysine; MEM, maximum entropy method; DOPC, 1,2-dioleoyl-*sn*-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-phosphoserine.

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ANX A3 belongs to a class of water-soluble proteins widely distributed in different species, tissues, and cell types (Seaton and Dedman 1998). This particular protein is expressed almost exclusively in differentiated cells of the myeloid cell lineage (Coméra et al. 1989). During the differentiation of these cells, ANX A3 can represent up to 1% of the cytosolic proteins (LeCabec et al. 1992). It has been found in the phagocytic pathway. During uptake of opsonized yeast by neutrophils, this protein becomes translocated from a wide cytoplasmic distribution to the close vicinity of phagosomes (Ernst 1991). In resting neutrophils and monocytes, it is associated with cytoplasmic granules and translocates to the membranes in activated cells (LeCabec and Maridonneau-Parini 1994). It promotes the aggregation *in vitro* of isolated specific granules in the presence of 1 mM Ca^{2+} . The protein can be efficiently phosphorylated *in vitro* by PKC on residue T11 in the N-terminal segment (Rothhut 1997).

Binding to negatively charged phospholipids and specific cellular membranes in a Ca^{2+} -dependent manner, and associated structural and dynamic changes are likely the primary events of the action of this protein on membrane trafficking (Meers 1996). ANX A3 exhibits large structural homologies (Favier-Perron et al. 1996) with other annexins and particularly with ANX A5 (Huber et al. 1992). The four conserved domains of about 70 amino acids each correspond classically to structural domains (named domain I–IV), comprising five α -helices (named A–E), wrapped into a right-handed super-helix (Favier-Perron et al. 1996). One principal calcium-binding site is present in each structural domain, situated on the convex face of the molecule. The N-terminal sequence of ANX A3 is however longer than that of ANX A5 and contains 20 amino-acid residues, instead of 17 for the latter. ANX A3 contains two tryptophan residues that can be used as reporter: the first one lies in the N-terminal segment (W5) and a second at the extremity of the IIIA–IIIB loop (W190), in an equivalent position to the single Trp residue of human ANX A5 (W187).

This study investigates the structure and dynamics of these two regions of ANX A3 and their changes in response to Ca^{2+} and membrane binding. This is especially important for the N terminus as much less is known for this domain (Arboledas et al. 1997; Marriott et al. 1990; Ayala-Sanmartin et al. 2000b) than for domain III, which undergoes large conformation transition in several annexins in response to both Ca^{2+} and membrane binding (Meers 1990; Meers et al. 1991; Meers and Mealy 1993a; Sopkova et al. 1993, 1994; Follenius-Wund et al. 1997; Sopkova et al. 1999; Ayala-Sanmartin et al. 2000b). For this study, two single-point mutants containing only one tryptophan residue each, the other being replaced by alanine, were studied (mutants ANX A3-W190A and ANX A3-W5A). The first mutation was expected to preserve the protein structure as did the W185A mutation performed for rat ANX A5 in domain III (Campos et al. 1998). The second mutation W5A, on the

other hand, was chosen to affect the ionic channel activity (Hofmann et al. 2000). It suppresses stabilizing interactions in the short N terminus as compared to the wild-type protein (Favier-Perron et al. 1996), but the rest of the structure remains unchanged. Because the protein does not crystallize in the absence of Ca^{2+} , a mutant lacking the principal Ca^{2+} binding site of domain III, in which the bidentate Ca^{2+} ligand E231 was replaced by an alanine, was constructed and its three-dimensional structure solved to provide a reference structure without Ca^{2+} bound in domain III.

The results show that in solution in the absence of Ca^{2+} , the W190 residue is exposed to the solvent on the protein surface, in agreement with the three-dimensional structures of the ANX A3 wild type (Favier-Perron et al. 1996) and of the ANX A3-E231A mutant, in contrast to W187 in ANX A5 (Huber et al. 1992; Sopkova et al. 1994), but similar to the ANX A5-D226K mutant (Sopkova-De Oliveira Santos et al. 2001). Binding of the protein to negatively charged membranes not only strongly affects the dynamics of W190 in domain III, but also W5 in the N-terminal segment, although to a lesser extent. With respect to the ANX A3 wild type, the W190A mutation increases significantly the Ca^{2+} concentration for half binding of the protein to the membranes, while the W5A mutation decreases it. These observations give additional experimental support to the recent suggestion about the role of the N-terminal domain of ANX A3 in the modulation of membrane binding and ion permeation by ANX A3 (Hofmann et al. 2000).

Results

Crystal structure of the ANX A3-E231A mutant

The crystal structure of mutant ANX A3-E231A (Fig. 1A) shows some interesting features compared to the crystal structure of wild-type ANX A3 (Fig. 1B), although their overall fold is very similar. Especially in domain III, where no calcium-binding site is occupied, the A-B loop and consequently the entire domain III becomes extremely mobile compared to the wild-type protein (Favier-Perron et al. 1996). The domain III mean B factor of the mutant is about 20 Å² higher than for the wild-type structure. Even though the extremity of the IIIA–B loop (residues 190–191) was refined with 0.5 occupancy, we observed a continuous density along the entire loop. The extremity of this loop adopts a conformation somewhat different from that of the wild-type structure (Fig. 1C and 1D, respectively) (Favier-Perron et al. 1996). The W190 residue situated in its extremity is turned toward the center of domain III. In the E231A structure, one of the secondary calcium-binding sites in the IIID–E loop is occupied by a water molecule. The presence of this molecule makes the conformation of the D–E loop rigid, in a position similar to that observed when the primary

Fig. 1. Ribbon representation of the overall folding of the ANX A3-E231A mutant (A) and of the ANX A3 wild type (B). Detailed structure of domain III of ANX A3-E231A mutant (C) and of the ANX A3 wild type (D). The calcium ions are shown as red balls, W5, W190, E231, and K229 residues are shown in balls and sticks. Arrows point to the N-terminal segment.

calcium-binding site is formed, and thus probably favors the exposure of the IIIA-B loop at the surface of protein.

Another interesting feature is the different position of the N terminus in the mutant structure. Residues 1–5 lie in the central channel between helices A of domain II and B of domain IV in the mutant (Fig. 1A), while this segment in the wild-type protein is devoid of contact with the rest of the molecule (Fig. 1B). This could be a reason for different crystal packing of the mutant as compared to the wild-type protein. There is enough space, however, between the neighboring molecules in the crystal to accommodate a conformation of the N terminus of the mutant similar to that of the wild type.

The environment of the tryptophan residues in the wild type and in single-tryptophan ANX A3 mutants (ANX A3-W5A and ANX A3-W190A) in solution

The steady-state fluorescence emission spectrum of the wild-type ANX A3 in the absence of Ca^{2+} and membranes

is characterized by an emission maximum at ~ 343 nm. The measurements carried out on the corresponding single Trp mutants at the same concentration (W190A and W5A) reveal clearly that the fluorescence emission of the wild-type protein is dominated by the signal of the N-terminal tryptophan (W5). The relative fluorescence intensity of the W5 residue is ~ 5 times higher than that of the W190 residue. The maximum of the emission spectrum of the latter in the W5A mutant lies at 340 nm, slightly blue-shifted with respect to that of the wild-type protein and of the W5 residue in the W190A mutant (342 nm), but 15 nm red-shifted as compared to the spectrum of the W187 residue in wild-type ANX-A5 in the absence of Ca^{2+} (Sopkova et al. 1994). This suggests that the W190 residue in ANX A3 is more exposed to the solvent than W187 in ANX A5, in agreement with the respective three-dimensional structures of these two proteins (Huber et al. 1992; Favier-Perron et al. 1996).

The fluorescence intensity decay of the wild-type protein is multiexponential. Four lifetime populations are detected

by MEM analysis of the data (Fig. 2A and Table 1). Two major lifetime populations dominate the decay. The shortest one characterizes the fluorescence intensity decay of the W190 residue in the W5A mutant (Fig. 2B), while the longest one is because of the W5 emission, as shown by the lifetime distribution of the W190A mutant (Fig. 2C). The lifetime distribution of the wild-type ANX A3 can be reconstructed from the distribution of each Trp residue in the single-Trp mutants, taking into account their relative quantum yields (0.05 and 0.23, respectively for W190 and W5) (Fig. 2D). This shows that none of the mutations affect the environment of the Trp residues.

Fig. 2. MEM recovered excited-state lifetime distribution of (A) ANX A3 wild type, (B) ANX A3-W5A mutant, (C) ANX A3-W190A mutant, (D) plain line: wild type; dotted line: weighted addition of the lifetime distribution of the two single-Trp mutants.

Table 1. Fluorescence intensity decay parameters obtained by MEM of the tryptophan fluorescence emission in annexin 3 wild type and in the single Trp mutants W5A and W190A

Proteins	τ_1 (ns) ^a α_1 ^b	τ_2 (ns) α_2	τ_3 (ns) α_3	τ_4 (ns) α_4	$\langle\tau\rangle$ (ns) ^c
ANX A3	0.16 0.33	0.83 0.14	2.85 0.10	5.57 0.43	2.82
ANX A3 10 mM CaCl ₂	0.15 0.13	0.89 0.14	2.19 0.16	5.48 0.57	3.62
ANX A3 L/P-200 10 mM CaCl ₂	0.15 0.16	0.99 0.04	2.53 0.14	5.75 0.66	4.21
ANX A3 W5A mutant	0.19 0.52	0.76 0.26	2.70 0.16	5.52 0.06	1.05
ANX A3 W5A 1 mM CaCl ₂	0.17 0.27	0.97 0.29	2.95 0.29	4.93 0.15	1.92
ANX A3 W5A L/P-200 10 mM CaCl ₂	0.32 0.06	1.59 0.22	—	5.63 0.72	4.50
ANX A3-W190A	0.09 0.03	0.37 0.05	1.82 0.07	5.74 0.85	5.01
ANX A3 W190A mM CaCl ₂	0.13 0.15	—	1.24 0.08	5.73 0.77	4.56
ANX A3 W190A L/P-200 10 mM CaCl ₂	—	0.41 0.09	1.23 0.04	5.56 0.87	4.98

The fluorescence intensity decays $T(t)$ are reconstructed from the parallel and perpendicular polarized components $I_{vv}(t)$ and $I_{vh}(t)$ such as $T(t) = I_{vv}(t) + 2\beta_{\text{corr}}I_{vh}(t) = \int_0^\infty \alpha(\tau) \exp(-t/\tau) d\tau$. τ is the excited-state lifetime, $\alpha(\tau)$ is its amplitude, β_{corr} is a correction factor accounting for the difference in transmission of the $I_{vv}(t)$ and $I_{vh}(t)$ components. Excitation wavelength: 295 nm; emission wavelength: 340 nm.

^a τ_i and ^b α_i are respectively the values of the center and of the normalized amplitude of each lifetime peak.

^c The mean lifetime $\langle\tau\rangle$ is calculated as: $\langle\tau\rangle = \sum_i \alpha_i \tau_i$.

The difference in solvent accessibility of the Trp residues can be quantified by time-resolved, acrylamide-quenching experiments. The results are represented by linear Stern-Volmer plots (Fig. 3). The W5 residue in the W190A mutant displaying one major lifetime is characterized by a relatively low value of the Stern-Volmer quenching constant K_{sv} and a corresponding low bimolecular quenching constant, k_q (Table 2). The W5 residue therefore is not easily accessible to the solvent (Eftink 1991), which is compatible

Table 2. Acrylamide quenching parameters for the Trp residues in the single-Trp mutants W5A and W190A

Residue number	Lifetime (ns) without acrylamide	K_{sv} (M ⁻¹)	k_q (M ⁻¹ s ⁻¹)
W5	5.74	2.4	$4.2 \cdot 10^8$
W190	0.76	3.72	$6.7 \cdot 10^9$
W190	2.76	8.22	$3.5 \cdot 10^9$
W190	5.52	4.59	$8.8 \cdot 10^8$
W190	1.05 ^a	4.50	$4.02 \cdot 10^9$

The Stern-Volmer constant values K_{sv} were obtained from linear plots of τ_0/τ_{10} as a function of acrylamide concentration. Regression coefficient values ranged from 0.899 to 0.996. Optical conditions as in Table 1.

^a Mean excited state lifetime defined as in Table 1.

Fig. 3. Stern-Volmer representation of the time-resolved acrylamide quenching of W5 and W190 in the single-Trp mutants of ANX A3. (○) τ_4 of the W190A mutant; (●) τ_2 of the W5A mutant; (◆) τ_3 of the W5A mutant; (▲) τ_4 of the W5A mutant. Optical conditions as in Table 1.

with the three-dimensional structure of the protein (Favier-Perron et al. 1996). A relative accessibility of ~5% can be estimated (Johnson and Yguerabide 1985) from the value of the bimolecular quenching constant of N-acetyltryptophanamide ($6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) (Eftink 1991) and is in agreement with the accessibility estimated on the basis of the X-ray structure of both the wild type and the E231A mutant (4.3% and 4.6%, respectively).

The quenching parameters for the W190 residue in the W5A mutant are less easily obtained as the fluorescence decay of this residue is highly heterogeneous (Table 2). The data for the major short lifetime are too scattered to obtain a reliable K_{sv} value. The three other lifetime populations are significantly quenched by acrylamide and reliable data for each of them are obtained with linear Stern-Volmer representations (Fig. 3). Much larger k_q constants are observed for residue W190 than for W5 (Table 2). Differences are, however, observed between the k_q constants for the individual lifetime classes. The longest lifetime displays a bimolecular quenching constant value significantly smaller than the shorter ones. This indicates that the lifetime populations might correspond to ground state conformers of W190, the longest lifetime being associated with the least solvent-accessible one. From the mean lifetime, we can however conclude that residue W190 is much more accessible to the solvent than W5 (Table 1). An accessibility to the solvent of ~70% is deduced within the range of the values computed from the crystal structure (78% and 66% for ANX A3 and E231A mutant, respectively). By comparison, the bimolecular quenching constant value for W187 in the wild-type ANX A5 in the buried conformation in the absence of Ca^{2+} was $4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, while that in the open conformation was $1.3\text{--}1.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Sopkova et al. 1998; Sopkova-De Oliveira Santos et al. 2001).

Mobility of the Trp residues in the ANX A3 wild type and its single-Trp mutants: fluorescence anisotropy decays

The experimental fluorescence anisotropy decay of the wild-type protein exhibits a fast initial depolarization followed by a slower one (not shown). The analysis by a one-dimensional model in which each lifetime τ_i is associated to every rotational correlation time θ_i (Ayala-Sanmartin et al. 2000b) allows the detection of two rotational correlation times: a first one in the subnanosecond time scale and a major second one in the nanosecond time scale (Table 3). The subnanosecond correlation time describes average internal rotation over the two Trp residues (dominated by the strongly fluorescent residue W5), whereas the second one is most likely the result of the Brownian rotational motion of the protein. It is of the same order of magnitude as in ANX A5 (Sopkova et al. 1994, 1999).

The rotational motions of each individual Trp residue in the wild-type protein can, however, be obtained by a two-dimensional analysis of the polarized fluorescence intensity decays (Rouvière et al. 1997; Sopkova et al. 1998, 1999; Ayala-Sanmartin et al. 2000b; Li de La Sierra et al. 2000). The results of the two-dimensional analysis of the wild-type polarized decays, represented as a (τ , θ) map, show

Table 3. Fluorescence anisotropy decay parameters for Trp residues in ANX A3 wild type and the two single-Trp mutants (W5A and W190A) in different experimental conditions

Sample	CaCl ₂ (M)	L/P	θ_1 (ns) β_1	θ_2 (ns) β_2	θ_3 (ns) β_3	$A_{t=0}$ $\omega_{\max}$ (°)
ANX A3	0	0	0.4	—	19.8	0.190
			0.025	—	0.165	20
	0.01	0	—	—	19.8	0.130
W5A	0.01	200	0.3	2.8	∞	0.161
			0.026	0.016	0.119	29
	0	0	—	1.1	19.5	0.168
W190A	0.01	0	—	2.0	8.2	0.100
			—	0.054	0.046	38
	0.01	200	0.2	9	∞	0.180
			0.043	0.027	0.110	28
	0	0	—	1.3	21.3	0.142
			—	0.007	0.135	27
	0.01	0	—	—	14.9	0.113
			—	—	0.113	35
	0.01	200	—	2.5	∞	0.142
			—	0.004	0.138	27

The fluorescence anisotropy is described by a sum of exponentials: $A(t) = \sum \beta_i \exp(-t/\theta_i)$ with $A_{t=0} = \sum \beta_i$. A value of the intrinsic anisotropy A_0 of 0.2 (Valeur and Weber 1977) was used for the calculation of the wobbling-in-cone semiangle $\omega_{\max}$ of the indole rotation using the β_i coefficients associated with the nanosecond correlation times (Vincent and Gallay 1991), according to Kinoshita et al. (1977): $\sum \beta_i/A_0 = [1/2 \cos \omega_{\max} (1 + \cos \omega_{\max})]^2$. Optical conditions as in Table 1.

Fig. 4. (τ, θ) maps obtained by MEM analysis of the polarized fluorescence decays of the Trp residues in ANX A3 wild type (A); ANX A3-W5A mutant (B) and ANX A3-W190A mutant (C). The analysis was performed on the polarized fluorescence intensity decays $I_{vv}(t)$ and $I_{vh}(t)$ using the classical expressions: $I_{vv}(t) = \frac{1}{3} \int_0^\infty \int_0^\infty \Gamma(\tau, \theta) e^{-t/\tau} (1 + 2Ae^{-t/\theta}) d\tau d\theta$ and $I_{vh}(t) = \frac{1}{3} \int_0^\infty \int_0^\infty \Gamma(\tau, \theta) e^{-t/\tau} (1 - Ae^{-t/\theta}) d\tau d\theta$. $\Gamma(\tau, \theta)$ is the relative proportion of emitter with lifetime τ and correlation time θ , A is the intrinsic anisotropy taken as 0.2 (Valeur and Weber 1977).

two main peaks (Fig. 4A). The first one associates the shortest lifetime (characterizing the W190 emission) with a 0.9–1-ns rotational correlation time, and the second major peak associates the longest lifetime (characterizing the W5 emission) with a long rotational correlation time of 15 ns, corresponding to the Brownian rotation of the protein.

The mobility of each Trp residue also was measured on the single-Trp mutants of ANX A3. Two rotational correlation-time populations describe the fluorescence anisotropy decay of the W190 residue in the W5A mutant (Table 3). The long rotation correlation time displays the same value as in the wild-type protein and describes the Brownian motion of the entire protein. The fastest detectable motion occurs in the nanosecond time range, like the result of the two-dimensional analysis of the wild-type protein. Subnanosecond motions not measurable with our instrumentation also may be present because the initial anisotropy value $A_t = 0$ is lower than the value of 0.2 expected at the excitation wavelength of 295 nm (Valeur and Weber 1977) (Table 3). We have also performed a two-dimensional analysis of the W5A mutant. The (τ, θ) map shows a major peak corresponding to the major short lifetime associated with the ~1 ns rotational motion (Fig. 4B).

The fluorescence anisotropy decay of the W5 residue in the W190A mutant also is described by a bimodal distribution. The long rotational correlation time displays the expected value for the Brownian rotation of the protein (Table 3). The nanosecond rotation is of small amplitude. As for W190, the $A_t = 0$ for W5 is however low, which indicates the existence of subnanosecond rotational motion. The semi-angle ω_{max} of the wobbling-in-cone subnanosecond rotation of W5 is larger than that of W190 (Table 3). The two-dimensional analysis of the polarized fluorescence decays of this mutant also was performed. The resulting (τ, θ) map exhibits two major peaks associated with the main long lifetime characterizing the W5 emission (Fig. 4C): a subnanosecond motion and a nanosecond rotation of ~15 ns.

Therefore, the data for the mutants allow recovery of the major trends of the (τ, θ) map for the ANX A3 wild type, such as, two major peaks associating on one hand the short lifetime of the W190 residue with a faster rotation and the long lifetime of the W5 residue with the Brownian rotation of the protein. This shows that the mutations do not affect the mobility of the remaining Trp residue.

Effect of Ca^{2+} binding on the conformation and dynamics of the N-terminal and domain III regions

The addition of calcium to the W190A mutant does not have any effect on the maximum of the fluorescence emission spectrum of the W5 residue (not shown). The time-resolved fluorescence decay analysis shows that the effect of calcium addition on the conformation of the N-terminal region of the

protein (Table 1) is small. The mobility of the W5 residue is, however, significantly increased by Ca^{2+} binding, as shown by the larger value of the semiangle of the wobbling-in-cone subnanosecond motion $\omega_{\max}$ (Table 3).

Because W190 is already partially exposed to the solvent in the absence of calcium, the effect of calcium ions on its steady-state fluorescence emission spectrum is less spectacular than that observed for W187 in the case of ANX A5 (Sopkova et al. 1994). Nevertheless, in the presence of a high calcium concentration (20 mM), the emission maximum of the W5A mutant is red-shifted to 347 nm. This is identical to our observations on the ANX A5 D226K mutant (Sopkova-De Oliveira Santos et al. 2001). The half-maximum effect on the red shift occurs at a calcium concentration of about 10 mM (not shown). A red-shift of 9 nm is induced by decreasing the pH to 4 (not shown).

The time-resolved fluorescence intensity decay of the W190 residue is also significantly modified by addition of 10 mM calcium. The amplitude of the shortest lifetime population is decreased to the profit of the longest lifetime (Table 1 and Fig. 5A,B). These changes show that Ca^{2+} binding in domain III favors this conformer. Simultaneously, the W190 mobility strongly increases upon calcium binding as shown by the large increase of the wobbling-in-cone semiangle value $\omega_{\max}$ (Table 3).

Effect of membrane binding on the conformation and dynamics of the N-terminal and domain III regions

In the presence of phospholipid vesicles, the excited state lifetime distribution of the W5 residue in the W190A mutant remains almost unchanged. It is still dominated by a long lifetime (Table 1). The local conformation remains therefore unchanged. Its mobility is nevertheless slightly affected by membrane binding as compared to Ca^{2+} alone. The fluorescence anisotropy decay shows the existence of a plateau value of the anisotropy (A_{∞}), which indicates that the protein is firmly bound to the membranes (the β_3 term in Table 3). A fast rotation of the W5 indole ring still occurs with reduced amplitude as compared to Ca^{2+} only. The semi-angle of the wobbling-in-cone rotation $\omega_{\max}$ is decreased to its initial value in the absence of Ca^{2+} and membranes (Table 3).

The Ca^{2+} -dependent binding of the W190A mutant to negatively charged membranes was monitored by steady-state fluorescence anisotropy. The half-maximum effect occurs at ~ 3 mM Ca^{2+} , higher than that for ANX A3 wild type, which occurs at ~ 1 mM Ca^{2+} (Fig. 6).

The interaction of the W5A mutant of ANX A3 with negatively charged phospholipid membranes is characterized by a gradual increase of the amplitude of the longest excited-state lifetime of W190 as a function of phospholipid/protein ratio in the presence of 10 mM Ca^{2+} . At lipid saturation, it represents ~ 70 – 75% of the fluorescence decay

Fig. 5. MEM recovered excited-state lifetime distribution of the W190 residue in the W5A ANX A3 mutant: in buffer (A), in the presence of 10 mM Ca^{2+} , and in the presence of 10 mM Ca^{2+} and lipid vesicles (L/P = 200) (C). Optical conditions as in Table 1.

components of W190 (90% of the fluorescence intensity) (Table 1; Fig. 5C). This lifetime value is close to that of W187 in ANX A5 in its complex with membranes (Sopkova et al. 1999). The Ca^{2+} half-maximum effect on the amplitude increase occurs at a lower Ca^{2+} ion concentration (0.5 mM) than for ANX A3 wild type (Fig. 6).

The mobility of the W190 indole ring is affected upon binding of the W5A protein to the membrane. The amplitude of the subnanosecond rotational motions (the semi-angle of the subnanosecond wobbling-in-cone rotation $\omega_{\max}$) decreases as compared to that in the presence of Ca^{2+} only, but increases as compared to the protein alone (Table 3). The nanosecond motion is slowed down and its amplitude is reduced.

Discussion

The present results shed light on the structure and dynamics of two distinct regions of ANX A3, its domain III and its

Table 4. Model refinement statistics of ANX 3-E231A

Parameter	
Resolution (Å)	10.0–2.3
Number of reflections used for refinement	15243
Number of	
Nonhydrogen protein atoms	2569
Calcium ions	3
Solvent molecules	142
R-factor (%)	19.8
R-free (%)	25.3
Mean B-factor	
Protein and calcium atoms (Å ²)	34.44
Solvent molecules (Å ²)	44.50
R.M.S. deviation from target values	
Bonds (Å)	0.010
Planes (Å)	0.012
Torsion (°)	3.0

N-terminal region and their responses to Ca^{2+} and membrane binding. We observed that the W5 residue dominates strongly the fluorescence emission of the wild-type protein. Single Trp mutants of the protein were therefore required to observe the W190 fluorescence emission. The comparison of the fluorescence properties of the two Trp residues in the wild type and in the two mutants shows that none of these mutations affect the environment of the remaining Trp residue. Therefore, both Trp residues report specific information on their environments and on their perturbations by Ca^{2+} and membrane binding, which are more explicitly measured in the single Trp mutants than in the wild-type protein. The data show that domain III exhibits different structural and dynamic properties as compared to the highly homologous protein ANX A5 and that Ca^{2+} and membrane binding affect these two protein domains, although not to the same extent. They also show that these mutations affect the Ca^{2+} -dependent membrane binding properties of the protein.

Domain III of ANX A3 exists in a different conformation from that of ANX A5 (Favier-Perron et al. 1996). At 10 mM Ca^{2+} concentration, its crystal structure shows that the IIIA–IIIB loop lies on the surface of the protein, thus exposing the W190 indole ring to the solvent. The Ca^{2+} site is occupied. This open conformation also is observed here for the E231A mutant, but no Ca^{2+} ion is bound in the principal site of domain III. In the Ca^{2+} concentration conditions used for ANX3 A3, the W187 residue in domain III of ANX A5 was always found buried in the crystal structure (Huber et al. 1990; Concha et al. 1993; Sopkova et al. 1993). In solution, similar conclusions can be drawn from the fluorescence studies that show that the environment of W190 in ANX A3 is very different from that of W187 in ANX A5 in the absence of Ca^{2+} . Nevertheless, although the accessibility of W190 to the solvent is much larger than that of W187 in ANX A5 at neutral pH in the absence of Ca^{2+} (Sopkova et

al. 1994, 1998), its major short lifetime indicates that it is likely partially folded on the surface of the protein in such a way that its fluorescence emission can be partially quenched either by peptide bonds of the protein main chain (Chen et al. 1996) or by amino-acid side chains (Chen and Barkley 1998). According to the folded conformation observed in the crystal structure of the E231A mutant, the former type of quenching interactions are more likely, as there are no other strong quencher moieties close to the indole ring. The closest neighboring side chains are those of D193, E194, or D151, known to be weak quenchers (Chen and Barkley 1998). The efficiency of the electron transfer-quenching mechanism of the peptide bond decreasing exponentially with the distance between the indole $\text{C}_{\epsilon 3}$ atom and the carbonyl carbon of the peptide bond (Sillen et al. 2000; Ababou and Bombarda 2001) quite a moderate change in this distance in the different conformers can result in large effects on lifetime values and would explain the presence of short lifetimes. The differences in structure and dynamics of domain III in ANX A3 and ANX A5 also are reflected by the larger amplitudes of the local motions both in the subnanosecond and nanosecond time scales in the former than in the latter protein (Sopkova et al. 1999).

Domains III of ANX A3 and ANX A5 display therefore divergent behaviors despite their similar amino-acid sequences (46% identity in domains III). Several factors are known to destabilize the W187 solvent-buried conformation in ANX A5 and favor the solvent-exposed one like high Ca^{2+} concentrations (Sopkova et al. 1993, 1994), mild-acidic pH conditions (Beermann et al. 1998; Sopkova et al. 1998), interaction with phospholipid membranes (Follenius-Wund et al. 1997; Meers 1990; Meers et al. 1991; Meers and Mealy 1993b; Sopkova et al. 1999) and incorporation

Fig. 6. Relative effect of Ca^{2+} -dependent binding to membranes on the long lifetime proportion of the ANX 5 wild type (◆), of the ANX A5-D226K mutant (□) and of the ANX A3-W5A mutant (●). For the ANX A3-W190 mutant (■), the relative effect on the steady-state anisotropy is shown. L/P = 200. Optical conditions as in Table 1.

into reverse micelles in the absence of Ca^{2+} (Sopkova et al. 1999). The role of acidic amino-acid residues in ANX A5 was suggested by the mild-acidic pH effect (Beermann et al. 1998; Sopkova et al. 1998), by the molecular modeling of the pathway of the large-scale Ca^{2+} -induced conformational transition (Sopkova-De Oliveira Santos et al. 2000) and also by specific mutations (Sopkova-De Oliveira Santos et al. 2001). Examination of the acidic residue content in domain III for both proteins shows that they are highly conserved, in particular the sequence of 14 amino acids of the IIIA–IIIB loop centered on the W190 residue, which is fully conserved in both proteins. The IIID–IIIE loop, which also is important because the bidentate Ca^{2+} ligand was included in this sequence, is also conserved. A remarkable exception is D226 in ANX A5, which is replaced by K229 in ANX A3. Mutation of this residue D226 by a lysine (D226K mutant) in ANX A5 stabilizes the open form of domain III at neutral pH in the absence of Ca^{2+} (Sopkova-De Oliveira Santos et al. 2001). In ANX A3, the absence of this interaction could, in a similar way, stabilize the open form. The further red shift of the emission spectrum of the W190 residue observed at pH 4 suggests, however, that other acidic residues might be involved in the conformation of domain III.

Ca^{2+} , although not required to maintain the solvent-exposed conformation of W190, plays nevertheless an important role for the structure of its binding loop in domain III. This is shown by comparison of the crystal structures of the wild-type ANX A3 protein and that of the E231A mutant. In the latter, which does not contain any Ca^{2+} ion in domain III, the IIIA–B loop exhibits a much larger temperature factor than in the wild-type protein. In solution, the addition of Ca^{2+} to the ANX A3 W5A mutant up to 10 mM provokes a larger exposure to the solvent of W190, as indicated by a further red shift of the fluorescence emission spectrum. The increase in proportion of the shortest lifetime populations (corresponding likely to conformers not in contact with protein moieties) and the large increase in rotational dynamics of the indole ring (both in the subnanosecond and nanosecond domains) show that the W190 indole ring is more firmly moved onto the surface of the protein by Ca^{2+} binding.

The conformation and flexibility of domain III also are affected by binding to negatively charged phospholipid membranes in the presence of Ca^{2+} , but to a lesser extent than for ANX A5. In the latter protein, a large-scale conformational transition was observed leading to the exposure of W187 on the protein surface (Sopkova 1994; Sopkova et al. 1999). In ANX A3, no such large conformational change occurs as the W190 residue is already exposed at the protein surface in the absence of Ca^{2+} . Nevertheless, dynamic effects occur at the membrane surface: a major Trp conformer is selected that is more mobile than in the absence of Ca^{2+} and membranes. This can be compared to the effects observed for ANX A2 (W212) and ANX A5 (W187) in inter-

action with membranes (Sopkova 1994; Sopkova et al. 1999; Ayala-Sanmartin et al. 2000b). These Trp residues report, however, different regions of domain III, that is, the IIIA–IIIB surface loop for W187 and W190 (in ANX A5 and ANX A3, respectively) and the inner space between helices IIIA, IIIB, IIID, and IIIE for W212 (in ANX A2). This suggests that the interaction of these annexins with the membrane surface modifies significantly the conformation and dynamics of a large part of domain III and is not confined to the Ca^{2+} binding loop only. This conformational transition at the membrane surface could be involved in the formation of aggregates of ANX A3 as shown by cross-linking studies (Raguene-Nicol et al., in prep.) and of the trimer network in the case of ANX A5 (Oling et al. 2000).

In the N-terminal region of the protein, the spectroscopic characteristics of the W5 residue suggest a solvent-shielded location in a relatively polar cavity, with a subnanosecond mobility of larger amplitude than that of W190, but almost no nanosecond motion. These features are in agreement with the crystal structure of ANX A3 (Favier-Perron et al. 1996) and of the E231A mutant showing that the first residues of the N-terminal segment are located at the extremity of the central pore of the protein, in the hinge region between domains II and IV. The indole ring is in contact mostly with polar side chains. In particular, its N_ϵ atom forms a hydrogen bond with the D127 carboxylate group in α -helix IIC. The proximity of several α -helices, bearing large dipole moments (Hol 1985), creates a large electrostatic influence. None of these vicinal side chains are strong quenchers, however (Chen and Barkley 1998). Quenching of the indole fluorescence emission by peptide bonds also can occur by electron transfer (Chen et al. 1996). In the three-dimensional structure of the protein, no peptide bonds are present within a sphere of 5 Å around each atom of the indole ring; their quenching efficiency is therefore weak. This would explain the long, dominant, excited-state lifetime of W5.

Although situated at the opposite face of the principal Ca^{2+} binding site, the local mobility of W5 is increased by Ca^{2+} binding. The hinge region between the domains in the concave side becomes probably less compact, allowing a larger amplitude of rotation of the W5 residue. This effect of Ca^{2+} binding on the hinge motion of the putative central pore was postulated by molecular dynamic simulations of ANX A5 (Cregut et al. 1998). It was concluded that calcium binding to the protein led to a limitation of this hinge motion with more open conformations being favored, in agreement with our observations on W5 in ANX A3. Moreover, the interaction of the protein with the membranes reduces the W5 mobility back to its value in the absence of Ca^{2+} . The modular structure of the protein core is therefore flexible. Different conditions of the environment can affect the relative disposition of the four domains. Conversely, the re-

moval of the W5 residue facilitates the Ca^{2+} -dependent binding of the ANX3 W5A mutant to membranes.

The W190A mutant shows a Ca^{2+} -dependent binding to membranes requiring higher Ca^{2+} concentrations than for the wild type. This suggests that the tryptophan residue of domain III displays probably a role in the interaction of the protein with the membrane surface as it was shown for W187 in ANX A5 (Campos et al. 1998), although the present data as well as those on ANX A5 (Sopkova et al. 1999) do not support a strong interaction of these Trp residues with the lipid bilayer.

Conclusion

Despite the large set of available data, several gaps in our knowledge of the annexin-membrane interaction process remain to be filled. Most of the studies have been focused on the conserved core (Meers 1996; Gerke and Moss 1997; Seaton and Dedman 1998; Gallay et al. 2000), while a few have been focused on the effect of these proteins on the lipid moieties (Andree et al. 1992; Megli et al. 1998; Saurel et al. 1998; Cézanne et al. 1999). However, a growing set of experimental data suggest that the N terminus might represent a major regulatory element of specific effects associated with annexin-membrane interactions, like membrane fusion, permeability to ions, etc. Interaction of this segment with specific regions of the core may modulate their structure and dynamics. These effects can modify the function of the molecule. This can be particularly the case for long N-terminal-bearing annexins like ANX A2, which exhibits an extremely large increase of its sensitivity to Ca^{2+} for membrane aggregation, which reaches submicromolar concentration ranges, compatible with intracellular levels, when its N terminus is complexed to p11 (Ayala-Sanmartin et al. 2000b) or like ANX A1 (Bitto and Cho 1999). The recently solved three-dimensional structure of this last protein with its integral N-terminal segment (Rosengarth et al. 2001) shows a direct interaction of the extremity of this segment with the domain III of the core, where it folds as an α -helix, and which may act as a molecular shaft. It has been also shown that specific mutations of the PKC-phosphorylation sites of the N-terminal region of ANX A2 regulate the Ca^{2+} -dependent membrane aggregation by the core domain (Ayala-Sanmartin et al. 2000a). Shorter N-termini also may exhibit a role in membrane aggregation process like for ANX A4 (Kaetzel et al. 2001). ANX A3 does not induce any membrane aggregation *in vitro*, but the present data show that its N terminus might also play a role in the binding of the protein to the membrane, in agreement with a previous report (Hofmann et al. 2000). Conversely, the interaction of the protein with Ca^{2+} and membranes modifies the conformation and dynamics of the N terminus, as it was also observed for ANX A1 (de la Fuente and Ossa 1997). This work suggests that the core and the N-terminal seg-

ment of ANX A3 (and especially W5) might be functionally interrelated. A hinge movement provoked by binding to membranes might cause a variation of the intermodule angle and might open the calcium ion path. Therefore, these results provide additional supports to the conclusion about the influence of the N-terminal segment on the cation permeation of the membranes provoked by this protein (Hofmann et al. 2000).

Materials and methods

Chemicals

Phospholipids (DOPC and POPs) were obtained from Avanti Polar Lipids. All other chemicals were of analytical grade purity and obtained from Merck.

Site-directed mutagenesis

Annexin A3 cDNA was obtained from a pUC plasmid (BIOGEN) and previously cloned into a pGEX-2T vector (Pharmacia) or a Bluescript II KS+ vector (Hofmann et al. 2000). The E231A mutation was introduced in the KS+/ANX plasmid by oligonucleotide-directed mutagenesis using the procedure of Kunkel (1985) with the synthetic oligonucleotide 5'-pGAC AGC ATA AAA GGA GCA TTA TCT TGG C-3'. The mutated annexin A3 cDNA insert was purified by agarose gel electrophoresis and cloned again into the pGEX-2T vector. The cDNA of the mutants were sequenced in their full length to verify the presence of the desired mutation (Hofmann et al. 2000). The W190A mutation was introduced in the pGEX-anx3 plasmid with the QuikChange site-directed mutagenesis kit (Stratagene) using the following synthetic oligonucleotide: 5'-pGGT GAG AAC AGA TAT GGC ACG GAT GAA GAC-3'. The cDNA of the mutant was sequenced in its full length to verify the presence of the desired mutation.

Preparation of proteins

The pGEX vector allows the production of a Glutathione *S*-transferase fusion protein. One-liter-culture of *Escherichia coli* BL21 gold (Stratagene) transformed with the appropriate plasmid was induced with 0.2 mM IPTG when the absorbance reached 0.9 U.A. at 600 nm. After an additional 3 h of culture, bacteria were lysed in 30 mL TENG (Tris pH 7.4 50 mM, EDTA 1 mM, NaCl 100 mM, NP40 1%, glycerol 10%, DTT 1 mM, lysozyme 0.5 mg/mL) supplemented with protease inhibitors (aprotinin 2 $\mu\text{g/mL}$, PMSF 1 mM, leupeptin 2 $\mu\text{g/mL}$, pepstatin 2 $\mu\text{g/mL}$, trypsin inhibitor 40 $\mu\text{g/mL}$). Lysis is achieved by mild sonication. The soluble fraction was purified on a 10-mL column of agarose-glutathione (Sigma) where GST fusion proteins are retained. The cleavage of the ANX A3 moiety was carried out in Tris 50 mM pH 8, NaCl 150 mM by 1000 U thrombin for 1 h at 37°C with stirring. The sample was desalted into Tris 50 mM pH8, EDTA 1 mM and loaded onto a Resource Q (Pharmacia) column, then eluted with a NaCl gradient up to 0.5 M. After analysis on SDS-PAGE 10% acrylamide, the fractions were pooled and desalted into PIPES 20 mM pH 6.8.

For measurements of absorbance (protein concentration determination) and fluorescence, the protein solutions were prepared in 50mM Tris-HCl pH 7.5–8 buffer.

Preparation of phospholipid sonicated vesicles

The phospholipid suspensions were prepared by sonication using DOPC and POPS dissolved in chloroform. The organic solution was evaporated to dryness in a glass tube under a stream of nitrogen. Remaining traces of organic solvent were further removed by submitting the sample to high vacuum during several hours. Hydration of the sample was achieved with buffer and after vortexing, the multilamellar vesicles formed were sonicated at room temperature with the micro-tip of a Branson-B12 sonicator for 5 min with half-duty cycles. The POPS/DOPC mole fraction of the vesicles was 20%.

Fluorescence measurements

Fluorescence emission spectra were recorded between 300 and 420 nm (bandwidth 4 nm) on a SLM 8000 spectrofluorometer equipped with Hamamatsu photon counting detectors (model H3460–53) with an excitation wavelength of 295 nm (bandwidth 2 nm) using 5×5 or 10×10 mm² optical geometry cuvettes. To remove polarization-related artifacts, the fluorescence emission spectra were reconstructed from the four polarized spectra (Rouvière et al. 1997).

Fluorescence intensity and anisotropy decays were obtained by the time-correlated, single-photon counting technique from the $I_{vv}(t)$ and $I_{vh}(t)$ components recorded on the experimental set-up installed on the SB1 station on the synchrotron radiation source Super-ACO at Orsay, France (Vincent et al. 1995). The excitation wavelength was selected by a double monochromator (Jobin Yvon UV-DH10, bandwidth 4 nm). The emission monochromator was a Jobin-Yvon UV-H10. In the cases of measurements with membrane vesicles, a 1-M CuSO₄ solution cut-off filter in a 1-cm optical path quartz cell (cut-off: 305 nm) was placed in the emission optical path to suppress any elastic scattering from the lipid suspension. A microchannel plate photomultiplier tube Hamamatsu (model R3809U-02) was used as detector. Time resolution was ~20 ps, and the data were stored in 2048 channels. Automatic sampling cycles including 30 s accumulation time for the instrumental response function and 90 s acquisition time for each polarized component were carried out such that a total number of $2\text{--}4 \times 10^6$ counts was reached in the fluorescence intensity decay. When needed, blanks without protein were cumulated for the same period of time as the fluorescent sample (90 s) and subtracted. To reduce scattering artifacts, 5×5 mm² optical geometry quartz cuvettes were used. Analysis of the fluorescence intensity decays as sum of exponentials were performed by the maximum entropy method (MEM) (Brochon 1994). Details of the principle and of the application of the method to fluorescence decays previously have been published (Vincent et al. 1988). Analysis of the fluorescence polarized decays were performed by a one-dimensional model of the anisotropy (Vincent and Gallay 1991), which assumes that all the excited-state lifetimes are subjected to all the rotational motions and by a two-dimensional (τ , θ) model, which allows to describe the coupling between lifetime and rotational correlation time (Rouvière et al. 1997; Sopkova et al. 1998, 1999; Ayala-Sanmartin et al. 2000b; Li de La Sierra et al. 2000). This model starts without any a priori on the (τ , θ) coupling, as the MEM program can handle a large number of independent variables.

X-ray structure determination

Crystals of E231A mutant were obtained by vapor diffusion using PEG 6000 as a precipitant. The drop of 2 μ L contained 10 mg/mL,

2 mM CaCl₂, and 5–7.5% (w/v) PEG 6000 in 50 mM Tris-HCl buffer at pH 7. The drop was equilibrated against a well containing 10–15% PEG 6000 in the same buffer. The crystals were orthorhombic, space group P2₁2₁2₁, with cell dimension $a = 45.48$ Å, $b = 54.36$ Å, $c = 132.54$ Å, and with one molecule per asymmetric unit.

Diffraction data were recorded on the D41 station on the DCI synchrotron ring at LURE, equipped with an image-plate detector (MAR research, diameter 180 mm), using a wavelength of 1.4 Å and a crystal-to-detector distance of 120 mm. The data reduction was performed using DENZO (Otwinowski and Minor 1997) and the CCP4 program suite (Brick 1994). The crystal data characteristics are summarized in Table 4. Because the crystals of the E231A mutant are not isomorphous to those of wild-type ANX A3 (pdb ID 1AXN) (Favier-Perron et al. 1996), the initial phases were obtained by molecular replacement using the program AMoRE (Navaza 1994). The mutant model refinement was initiated with a few cycles of the restrained ARP procedure (Lamzin and Wilson 1993) followed by several rounds of alternate manual reconstruction on a graphics terminal with O (Jones et al. 1991) and refinement by REFMAC (Murshudov et al. 1997). Table 4 summarizes the final refined model characteristics.

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