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Conformational Adaptation of Annexin V upon Binding to Liposomes: A Time-Resolved Fluorescence Study

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The fluorescence intensity decay of the single tryptophan residue, Trp-187, of free annexin V is described by the sum of three lifetime components (5.4, 1.3, and 0.4 ns), which may be correlated to three ground-state classes of Trp conformers. The two major classes (44 and 48%) are embedded in the protein matrix. When annexin V binds to calcium and liposomes made of dioleoylphosphatidylcholine and dioleoylphosphatidylserine, similar results are obtained whatever the (10–200) lipid ratio. The Trp fluorescence decay is fitted with only two components (6.9–7.2 and 2.0–2.2 ns). Decay-associated spectra reveal that the longest lifetime of bound annexin V can be related to Trp residues (60%) located in a partially polar environment, which could correspond to the protein–membrane interface. The shortest lifetime is attributed to Trp residues (40%) which reside in a hydrophobic surrounding: these Trp residues would penetrate into the phospholipid membrane and contribute to the stabilization of the 2D-array of annexin V molecules.

Annexin V is a 35kDa protein which belongs to the annexin family of Ca^{2+}-dependent phospholipid binding proteins (for a review see 1-3). The *in vivo* role of these proteins is still unknown, but some antiinflammatory (4) or anticoagulant properties (5,6) have already been demonstrated. Annexin V also displays voltage-dependent ion channel activity (7,8).

The primary structure of this protein consists of 320 amino acid residues whose sequence shows a specific N-terminal tail and a core region of four highly-conserved repeats of 70 amino acid residues, implicated in the Ca^{2+}- and/or phospholipid-binding sites. The crystallographic studies of annexin V revealed an almost entirely α-helical structure (9-11), with the four domains arranged in an overall flat disc: on the concave side, there are both termini of the molecule, and, on the convex side, the Ca^{2+}-binding sites. Domain III contains the unique Trp-residue of annexin V, Trp-187, in a loop connecting helices IIIA and IIIB. It has previously been shown, by crystallography, that, in the absence of Ca^{2+}, the side chain of this residue is buried in the crystal (9), whereas it becomes exposed on the molecular surface in a high-calcium form of human annexin V (11) and in the rat annexin V structure (12).

The electron image analysis of two-dimensional crystals of annexin V bound to phosphatidylserine-containing layers showed a packing of annexin V molecules into trimers, all with the convex side facing the membrane (6,13-15). Ca^{2+}-dependent clustering of annexin V on lipid membranes has also been observed by chemical cross-linking (16).

Fluorescence spectroscopy studies (17-19) revealed that conformational changes of annexin V occurred upon Ca^{2+}-mediated binding to phospholipids, inducing a shift of Trp-187 from a hydrophobic to a more polar environment. Following the intrinsic steady-state fluorescence of Trp-187, we previously proposed a model for the formation of two-dimensional arrays of annexin V on the phosphatidylserine-containing liposomes (19). These arrays are stabilized by both i) specific binding between the annexin V and PS molecules of liposomes and ii) non-specific interactions between the annexin V molecules themselves. It was thus interesting to investigate, by time-resolved fluorescence spectroscopy, the properties and dynamics of the micro-environment of the unique Trp-187 of annexin V and to determine whether this residue could act as a reporter of two different conformations of annexin V, depending either on the specific "annexin V- PS" binding or on the

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Abbreviations: PS, dioleoylphosphatidylserine; PC, dioleoylphosphatidylcholine; HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethane sulfonic acid; EDTA, ethylenediaminetetraacetic acid; DMPC, dimyristoylphosphatidylcholine.

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"annexin V - annexin V" interactions. To fulfil this aim, the fluorescence of Trp-187 was followed in the presence of liposomes made of dioleoylphosphatidylcholine (PC) and dioleoylphosphatidylserine (PS) with concentration ratios chosen to fit precise macromolecular situations involving "annexin V-PS" and/or "annexin V-annexin V" interactions.

Our results clearly show that there is an equilibrium between different Trp conformers in free annexin V as well as in annexin V bound to liposomes and suggest that this residue may play a key role in the stabilization of the arrays of annexin V on PS-containing liposomes.

MATERIALS AND METHODS

Materials. Chemicals were of the best available commercial grade. Phospholipids (dioleoylphosphatidylcholine (PC), and dioleoylphosphatidylserine (PS)) were purchased from Avanti-Polar Lipids (Birmingham, Alabama, U.S.A.) and were used without further purification.

Annexin V was purified from human placenta according to Funakoshi et al. (20), with slight modifications as described previously (21).

Large unilamellar phospholipid vesicles with various compositions were prepared extemporarily from stock solutions at $3.5.10^{-3}$ M by extrusion on polycarbonate filters as (22). Phospholipid concentrations of the stock solutions and of the liposomes were determined according to the phosphorus assay by Bartlett (23). A total phospholipid concentration of 0.45 mM was used for each experiment, a concentration chosen so that the annexin V molecules had just the steric space to cover the liposome on one sheet, with an average diameter of 1200-1300 Å.

Spectroscopic methods. All measurements were performed, under continuous stirring, at 20°C on protein solutions at a concentration of 2.9 μM, in the following buffer : 10 mM Hepes, 150 mM NaCl, 0.02 % NaN_3, pH 7.4 (buffer A).The absorption spectra (Cary IV spectrophotometer) and the fluorescence spectra (Perkin-Elmer MPF-66 fluorometer) were obtained as described in (19).

For time-resolved fluorescence measurements, the excitation source was a pulsed, frequency-tripled titane-sapphire laser, pumped by a continuous argon laser (Spectra-Physics). The excitation wavelength was set at 295 nm, with a repetition rate of 4 MHz. The emission beam passed at right angle to the excitation beam through a polarizer set at the magic angle (54.7°) to the direction of the excitation polarization and then through a 4 nm band-pass monochromator (Jobin-Yvon H10) combined with a cutoff filter to eliminate the residual diffusion of the excitation light. The emitted photons were detected, by using the single photon counting method, by a Hamamatsu R3809U microchannel plates photomultiplier tube coupled to a Model 6954 pulse preamplifier (Phillips). The data were recorded on a multichannel analyzer (Ortec 921). Since no color effect of the microchannel plate was observed, the instrument response function was recorded with a polished aluminium reflector. Its full width at half-maximum was about 45 ps. For both the sample decay and the response function, the data accumulation rate was 3-5 kHz and the counting was stopped when the total number of counts reached 5.10^5 counts. The calibration was 25.5 ps/channel.

The decay data were transmitted to a workstation HP 9000-715/50 and analyzed as sums of exponentials:

$$I(t, \lambda) = I_0 \sum_i \alpha_i(\lambda)\, e^{-t/\tau_i(\lambda)}$$

where $I(t, \lambda)$ and I_0 are the intensities at time t and t=0, α_i are the normalized pre-exponential terms (or amplitudes), such as $\Sigma_i\, \alpha_i(\lambda) = 1$ and τ_i are the lifetime components. The analysis was performed using a new iterative reconvolution procedure based on the estimated covariance matrix (24). The main advantage of this method, based on the compound Poisson distribution is a reduced χ^2 value independent of the total number of counts, thus, avoiding the pitfall of artificially multiplying the number of exponential components in order to obtain a good deconvolution fit. This method was improved by multiplying the estimated variances by a corrective factor determined with a monoexponential decaying fluorophor as a standard (25). The number of exponentials in the fitting procedure was progressively increased until the fit did not ameliorate. The adequacy of the fit was judged by the value of reduced χ^2 and by visual inspection of the autocorrelation function and of the weighted residuals. In all cases, three components were necessary to describe the fluorescence decays, the third subnanosecond component significantly improving the fit. However, when annexin V bound to (PC/PS) liposomes, this component (with a lifetime < 100 ps) represented less than 1 % of the fluorescence intensity, and was therefore excluded from the data reported here. We only took into account the two main components, with their pre-exponential terms normalized to have a sum equal to 1.

The fractional intensities f_i of each species were calculated using

$$f_i(\lambda) = \alpha_i(\lambda)\tau_i(\lambda)/\sum_i \alpha_i(\lambda)\tau_i(\lambda)$$

Decay-associated spectra (DAS) were calculated from the steady-state fluorescence spectra and the multi-exponential analysis of the decay data at a set of wavelengths, regularly spaced, spanning the spectrum. The fluorescence associated to the component i at the wavelength λ was calculated using the formula (26):

$$F_i(\lambda) = F_{SS}(\lambda)\alpha_i(\lambda)\tau_i(\lambda)/\sum_j \alpha_j(\lambda)\tau_j(\lambda)$$

where $F_{SS}(\lambda)$ is the total intensity obtained from steady-state emission spectra.

RESULTS

1. Fluorescence Decay

Absorption and steady-state fluorescence properties of the unique Trp-187 of free and liposome-bound annexin V have already been described in detail (17-19). We checked that, in our experimental conditions, the Ca^{2+}-triggered binding of annexin V to the various liposomes was maximal: the fluorescence quantum yield of Trp-187 increased four fold (from 0.061 to 0.26) and the emission wavelength shifted from 327 to 338 nm. In this study, the time-resolved fluorescence of the Trp residue of annexin V bound to liposomes was compared to the decay of the free protein. As the Trp fluorescence intensity decays in peptides and proteins is most often described as a multi-exponential process (27-29), a discrete component analysis was used. Each component was defined by its lifetime τ_i and its pre-exponentional term or amplitude α_i (see Materials and Methods). From the α_i and τ_i values, we determined the fractional intensities, f_i, which represent the relative contribution of component i in the total fluorescence intensity. Table I shows the typical results obtained

TABLE I

Analysis of the 340 nm Fluorescence Intensity Decay of Annexin V in the Absence and in the Presence of (PC/PS) Liposomes of Various Ratios

	τ_1 (ns)	α_1	f_i (%)
Annexin V + EDTA	5.4 ± 0.1	0.08 ± 0.01	36 ± 1
	1.30 ± 0.03	0.44 ± 0.03	48 ± 1
	0.40 ± 0.03	0.48 ± 0.04	16 ± 1
Annexin V + (PC/PS=10)liposomes (pCa = 2.5)	7.2 ± 0.1	0.58 ± 0.03	82 ± 3
	2.2 ± 0.1	0.42 ± 0.03	18 ± 3
Annexin V + (PC/PS= 40)liposomes (pCa = 2)	6.9 ± 0.1	0.63 ± 0.05	85 ± 4
	2.0 ± 0.1	0.37 ± 0.05	15 ± 4
Annexin V + (PC/PS=200)liposomes (pCa = 1.9)	7.0 ± 0.2	0.60 ± 0.05	84 ± 5
	2.0 ± 0.1	0.40 ± 0.05	16 ± 5
Annexin V + PC liposomes (pCa = 2)	5.9 ± 0.1	0.20 ± 0.04	59 ± 2
	1.57 ± 0.05	0.42 ± 0.01	34 ± 2
	0.98 ± 0.02	0.38 ± 0.04	7 ± 1

Note. Lifetime components τ_i, normalized preexpontential terms α_i and fractional intensities f_i were expressed as means (± S.E.M.) for at least three independent experiments, corresponding each to 20 decays. The experiments have been carried out with solutions containing 2.9 μM annexin V in buffer A (see Materials and Methods) with Ca^{2+} concentrations corresponding to maximal annexin V binding. The excitation wavelength was set at 295 nm.

for the 340 nm fluorescence intensity decay of annexin V in the absence and in the presence of (PC/PS) liposomes of various ratios and calcium.

The fluorescence decay of *free annexin V* was adequately fitted with a minor long-lived component of 5.4 ns, an intermediate component of 1.3 ns and a short-lived component of 0.4 ns contributing respectively to about 36 %, 48 % and 16 % of the total fluorescence intensity at 340 nm. Some experiments were carried out with annexin V in the presence of Ca^{2+}. Up to pCa = 2, no change in steady-state fluorescence emission is observed and the decay analysis gives the same results as those obtained for free annexin V.

The decay of *liposome-bound annexin V* was fitted with only two components. The binding of annexin V to either type of (PC/PS) liposomes is expressed by an increase in both lifetime values (6.9 - 7.2 ns and 2.0 - 2.2 ns). Concomitantly, the contribution of the longer lifetime in the fluorescence intensity drastically increases (from 36 to 82-85 %) at the expense of the second lifetime contribution. As a similar distribution of τ_i and α_i values is observed, whatever the (PC/PS) composition of the liposomes tested, we can conclude that the Trp fluorescence properties are roughly independent of the (PC/PS) ratio. This suggests that i) annexin V undergoes a similar conformational change and ii) the structure of bound annexin V is the same, whatever it is involved in "annexin V-PS" binding or "annexin V-annexin V" interaction.

Comparative experiments were performed with annexin V in the presence of pure PC liposomes and Ca^{2+} (Table I). The decays were analyzed with three components, the α_i and τ_i values being comparable to those of free annexin V. As there is no increase in the fluorescence quantum yield, only a small shift of λ_{max} to 330 nm, we confirm our previous conclusions, that there is no real binding of annexin V to the liposomes, although the existence of some non-specific interactions between annexin V and PC cannot be excluded.

2. *Decay-Associated Spectra*

The fluorescence decay curves were recorded as a function of wavelength throughout the emission spectrum from 310 to 380 nm to get more information on the micro-environment of the Trp residue in annexin V, free or bound to (PC/PS) liposomes.

The spectra associated to each component of the decays (DAS) were calculated from the decay parameters and the steady-state spectra as described in Materials and Methods (Fig. 1). The fluorescence emission of *free annexin V* (λ_{max} = 327 nm) is predominantly due to the intermediate-lifetime component τ_2 (f_i = 48%, see Table I) whose emission maximum is around 325 nm (Fig. 1a) and, to a minor extent, to the short-lived component τ_3, which shows an emission maximum around 320 nm.

In the presence of Ca^{2+} and (PC/PS = 10) liposomes, there is an inversion of the contributions of the first two components, due to a large increase in τ_1 and in α_1 (Fig. 1b). The fluorescence emission of *liposome-bound annexin V* (λ_{max} = 338 nm) is largely dominated by that of the longer-lived component : i) its emission maximum (338-340 nm) is similar to that of the steady-state spectrum, ii) it participates, at 340 nm, to about 82 % to the total fluorescence intensity. Some comparable decay-associated spectra were obtained for annexin V bound to (PC/PS = 40) or (PC/PS = 100) liposomes (spectra not shown).

3. *Specific Classes of Trp Residues*

The lifetimes τ_i and the relative amplitudes α_i were approximately independent of the emission wave-

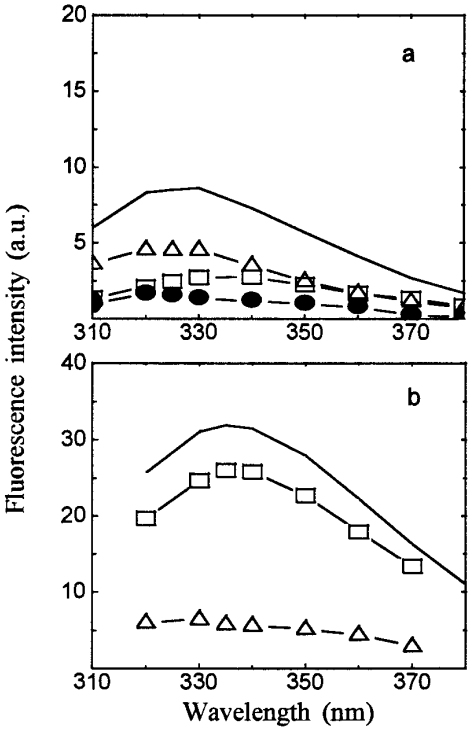

FIG. 1. Time-resolved fluorescence emission spectra associated with the long-lived component (□), the intermediate component (△) and the short-lived component (when present) (○) of Trp-187 fluorescence intensity decay in annexin V, (a) free and (b) bound to (PC/PS = 10) liposomes at pCa = 2.5. The solid line represents the steady-state fluorescence spectrum under the same experimental conditions.

length, as well in free as in bound annexin V. Therefore the exponential components recovered by data analysis are usually attributed to discrete classes of Trp residues, characterized by different local environments. The most common interpretation is that each class can be correlated to a specific rotamer (30). A schematic representation is given in Fig.2. The three interconverting classes ("a", "b" and "c") in free annexin V and the two classes ("d" and "e") in bound annexin V are characterized by ground-state equilibrium constants $K_{eq}^{xy} = x/y$ where x and y represent any class. Under the assumption that there is no interconversion between the classes at the excited state, the relative amplitudes α_i represent the respective concentrations of the ground-state classes. Thus, in the scheme of Fig. 2, each class is symbolized by a square whose area is proportional to its concentration, i.e. to α_i (cf Table I).

Free annexin V. A globally hydrophobic localization of Trp-187 has already been shown by steady-state fluorescence (17-19), and by crystallographic studies (10-12). However, this time-resolved fluorescence study allows us to quantify the different species. Indeed, classes "b" and "c", respectively associated to the two shortest lifetimes and representing 44 % and 48 % of the total concentration, are clearly dominant (Fig.2). Their related Trp residues lie in highly hydrophobic environments since their λ_{max} (325 and 320 nm) are close to the 325 nm emission maximum wavelength of N-acetyltryptophanamide in dioxane (31).

Liposome-bound annexin V. When annexin V binds to liposomes and Ca^{2+} (Fig. 2), the Trp residues can be divided into two classes, whose respective percentage is the same for the three (PC/PS) liposome ratios. There is a dominance of class "d" Trp residues (≈ 60 %), which lie in a more polar environment than class "e" Trp residues (≈ 40 %).

DISCUSSION

1. Liposome-Bound Annexin V Conformation

As previously shown (19), the two-dimensional arrays of annexin V on PS-containing liposomes were stabilized by both specific binding between annexin V molecules and PS molecules of liposomes and non-specific interactions between the annexin V molecules themselves. The conformational changes associated to both kinds of binding were explored by time-resolved fluorescence spectroscopy, using (PC/PS) liposomes of three various ratios. Assuming that annexin V and lipid molecules offer respectively areas of 25.5 and 0.7 nm^2 (13), i.e. one molecule of annexin V covers approximately 35-40 lipid molecules (32), each ratio corresponds to a different macromolecular situation:

i) For (PC/PS = 10) liposomes, each molecule of annexin V covers randomly four PS molecules and has the opportunity to bind up to four PS molecules by its homologous repeats, depending on the Ca^{2+} concentration. Thus, no major structural difference is likely to be observed between the annexin V molecules which must all be involved in the two types of bonds ("annexin V-PS" and "annexin V-annexin V"), the spectroscopic signal of the Trp residues only transducing one type of conformation.

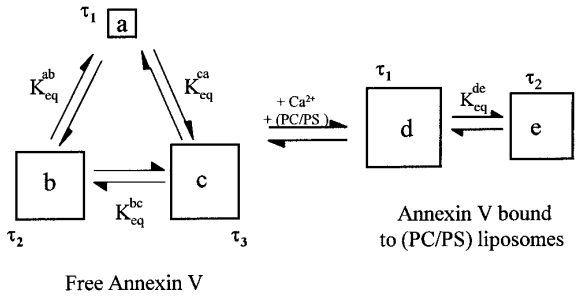

FIG. 2. Model of the Trp classes associated to the various lifetimes τ_i in annexin V, free or bound to (PC/PS) liposomes. According to the K_{eq} constants, the "a", "b" and "c" classes interconvert in free annexin V, whereas the "d" and "e" classes interconvert in annexin V bound to (PC/PS = 10, 40 or 200) liposomes. Each class is represented in a square whose area is proportional to its concentration, i.e. to the relative amplitude α_i (see Table I).

ii) By contrast, for (PC/PS = 200) liposomes, only some annexin V molecules can directly bind to one PS molecule - in average one out of five - and the major part of the array is constituted by annexin V molecules covering PC molecules and simply interacting together. In this latter case, there would be two types of annexin V populations which might be associated to two different conformations, with one of them being largely predominant.

iii) In the intermediate case, for (PC/PS = 40) liposomes, one annexin V molecule on average can bind to one PS molecule. The population of annexin V molecules would be homogeneous and present one conformation if each annexin V molecule bound to one PS molecule. However, if some annexin V molecules bound to more than one PS molecule, there would be some areas only constituted by annexin V molecules interacting together, and, consequently two types of annexin V populations with probably two conformations.

In the membrane-bound form of annexin V, our data clearly shows the existence of an equilibrium between two classes of Trp residues. However, the fact that the proportion of both classes of Trp does not vary with the (PC/PS) liposome ratio, leads us to conclude that there is not *one* conformation associated to the specific "annexin V - PS" binding and *another* to the non-specific "annexin V - annexin V" interaction. This rather suggests that there would be one array formed by annexin V molecules of a unique conformation, which is independent of the (PC/PS) liposome composition (in the range (PC/PS) = 10 to 200). Some molecules may be directly involved in the binding, with, as proposed by Sopkova et al. (11), in the third Ca^{2+}-binding site, the sulfate ion ligand replaced by a phosphoryl group and the water molecule ligand by the OH group of the serine headgroup of PS. The other molecules would be maintained on the liposome surface by essentially electrostatic interactions between annexin V molecules themselves or with the phospholipid molecules. Some hydrophobic interactions are not excluded but more improbable, since Trp-187 and its three structurally-related homologues (Leu-31, Ala-103, Ala-262) are the unique hydrophobic determinants on the otherwise hydrophilic membrane-facing surface of the protein (12).

2. Trp-187 Localization upon Annexin V-Membrane Interaction

The conformational change associated to the Ca^{2+}-dependent binding of annexin V to (PC/PS) liposomes globally results in a large dequenching of the Trp fluorescence, as revealed by the increase in lifetimes and fluorescence quantum yields(31). It is related to the transfer of Trp-187 from a protein hydrophobic pocket to a more polar localization (8). The existence of two different classes in bound-annexin V can be explained by different locations: the indole ring may either be inserted into the phospholipid bilayer or occupy a transitional region between the protein and the membrane or else alter its position within a pocket in the protein. In fact, taking into account the high Ca^{2+} concentration in the medium, the annexin V molecules may present the triclinic crystallographic structure described by Sopkova et al. (11), a structure where Trp is protruding out of the protein into the solvent. Such an external location of Trp-187 was also proposed for free annexin V in the presence of high Ca^{2+} concentrations in time-resolved fluorescence studies using the method of lifetimes distributions (33).

The results presented here allow us to propose that a large part of the Trp residues (60 %) lie at the interface between the polar headgroups of the phospholipid bilayer and the positively-charged residues of the protein backbone. This localization, characterized by a maximum emission wavelength at 340 nm, is comparable to the position of the partially-buried Trp residue in a pentagastrin structurally-related peptide interacting with DMPC vesicles (34). The other part of the Trp residues (40 %) may be in another conformation upon annexin V-liposome interaction and thus may have the opportunity to penetrate into the phospholipid bilayer, in agreement with the 325 nm maximum emission wavelength. This inclusion of Trp is consistent with the results obtained by Meers and Mealy (35), who showed that some Trp sidechains make contact with specific phospholipids in the membrane, at the level of the ester carbonyl oxygen and the first groups of the acyl chains. In either position, Trp becomes partially immobilized, but the insertion of Trp residues at some points would help to stabilize the annexin V array on the membrane surface.

Finally, the changes in the time-resolved fluorescence of Trp-187 upon annexin V-liposome binding lead us to conclude that Trp-187 plays an important role in the Ca^{2+}-dependent conformational adaptation of the annexin V molecules to the membrane. PS phospholipids may be necessary to recruit annexins to the membrane and initiate the formation of 2D-arrays of annexin V molecules. Subsequently, PS and/or Trp-187 would act as anchoring points of the array. The formation of tight Ca^{2+}-bridges between annexin V and the membrane, the Trp penetration into the phospholipid bilayer, associated to different electrostatic or hydrophobic protein-lipid interactions and lateral self-association of annexin V molecules by electrostatic bonds, all contribute to stabilize the array of annexin V molecules on the membrane.

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