Novel Organization and Properties of Annexin 2-Membrane Complexes*

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Annexin 2 belongs to the annexin family of proteins that bind to phospholipid membranes in a Ca2+-dependent manner. Here we show that, under mild acidic conditions, annexin 2 binds to and aggregates membranes containing anionic phospholipids, a fact that questions the mechanism of its interaction with membranes via Ca2+ bridges only. The H+ sensitivity of annexin 2-mediated aggregation is modulated by lipid composition (i.e. cholesterol content). Cryo-electron microscopy of aggregated liposomes revealed that both the monomeric and the tetrameric forms of the protein form bridges between the liposomes at acidic pH. Monomeric annexin 2 induced two different organizations of the membrane junctions. The first resembled that obtained at pH 7 in the presence of Ca2+. For the tetramer, the arrangement was different. These bridges seemed more flexible than the Ca2+-mediated junctions allowing the invagination of membranes. Time-resolved fluorescence analysis at mild acidic pH and the measurement of Stokes radius revealed that the protein undergoes conformational changes similar to those induced by Ca2+. Labeling with the lipophilic probe 3-(trifluoromethyl)-3-(m-[125I]iodophenyl)diazirine indicated that the protein has access to the hydrophobic part of the membrane at both acidic pH in the absence of Ca2+ and at neutral pH in the presence of Ca2+. Models for the membrane interactions of annexin 2 at neutral pH in the presence of Ca2+ and at acidic pH are discussed.

Annexin (Anx) 2 is a Ca2+-dependent phospholipid-binding protein found as a monomer of 38.5 kDa (AnxA2) and as a heterotetramer ((AnxA2-P11)2) when two monomers associate to a P11 dimer (1). AnxA2 is composed of two structural domains, the core and the N-terminal tail. The core domain contains four repeats (I–IV) of ~70 residues organized in a right-handed superhelix. Each repeat harbors a Ca2+-binding site on the convex face of the molecule (2). The N-terminal tail contains the P11 binding region (3–5). The role of AnxA2 is still not well understood, but the protein has been implicated in membrane-membrane and membrane-cytoskeleton interactions and, specifically, in the regulation of intracellular transport steps such as exocytosis and endocytosis (for reviews see Refs. 6 and 7).

The Ca2+-mediated binding of annexins to membranes at neutral pH is dependent on the presence of anionic phospholipids such as phosphatidylserine (PS) or phosphatidylinositol (8, 9). A large set of data shows that Ca2+-dependent binding also requires the presence of anionic phospholipids. Annexins 2, 5, and 6 extracted from matrix vesicles partition into organic phases at acidic pH (24), suggesting an increase in the hydrophobicity of the proteins. Annexins 1, 2, 5, 6, and 12 are able to bind to phospholipid membranes at mild acidic pH in the absence of Ca2+ (25–30). The EPR spectra of spin-tagged AnxB12 (28) and the reaction of the liposoluble molecule (3-trifluoromethyl)-3-(m-iodophenyl)diazirine (TID) with annexins 5 and 12 (29) suggested that these annexins became more hydrophobic at acidic pH. Langen et al. (28) proposed the change of two α-helices and their inter-

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1 The abbreviations used are: Anx, annexin; (AnxA2-P11)2, tetramer complex of annexin 2-P11; PS, phosphatidylserine; PC, phosphatidylcholine; cryo-TEM, cryo-transmission electron microscopy; MEM, maximal entropy method; [125I]TID, 3-(trifluoromethyl)-3-(m-[125I]iodophenyl)diazirine; Mes, 4-morpholineethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; MALDI-TOF MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometer.
helical hydrophilic loop into a transmembrane α-helix. The proposed conformational change of repeat II occurred only in the presence of membranes. However, this hypothesis is not consistent with experiments in which AnxA5 labeled by the fluorophore bis-1-anilino-8-naphthalenesulfonate at pH 7 or 5, either in the presence or absence of vesicles, showed similar fluorescence spectra, indicating the absence of macroscopic pH-dependent changes in the environment of the probe (25).

Here, the effect of acidification of the medium on the capacity of membrane binding and membrane aggregation, on the nature of the annexin 2-membrane junctions, and on the H⁺ phospholipid-induced conformational changes were investigated. Models for the H⁺-mediated membrane association and aggregation of annexin 2 are discussed.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Type III-B 1-α-phosphatidylcholine (PC), 1-α-phosphatidyl-l-serine (PS), BAPTA, bovine serum albumin, and ovalbumin were from Sigma. AnxA2 produced in Saccharomyces cerevisiae and P11 produced in Escherichia coli were purified as described (17). AnxA2 mutant L127W/P11 was obtained by mixing equimolar quantities of AnxA2 and P11. The AnxA2 mutant L127W/P11 was obtained by adding 125 I-labeled diazirine (1700 Ci/mmol) to the reaction mixtures and the samples were analyzed by SDS-PAGE. The pH was controlled using buffers A, pH 7, B (Mes 40 mM, pH 7), C (NaOH 0.3 M). Aggregation experiments were performed as described (17). In brief, aggregation was measured by the increase in the turbidity at 340 nm (26).

**Mass Spectrometry Experiments**

Reduction-Alkylation—Each annexin sample (~3 μg) was dissolved in 5 μl of 50 mM NH₄HCO₃ buffer, pH 8. The reduction step was performed with dithiothreitol. 10 mM dithiothreitol was added in 300 μl excess and incubated at 57 °C for 1 h. The alkylation step was performed with 350 μl excess of 55 mM iodoacetamide. The reaction was stopped after 15 min of incubation and the samples were centrifuged at the desired pH at 50 ml h⁻¹. The supernatant was discarded; the proteic suspension was 1 mg/ml. The reaction was stopped after 15 min of incubation and the samples were centrifuged at the desired pH at 50 ml h⁻¹. The supernatant was discarded; the proteic suspension was 1 mg/ml. The reaction was stopped after 15 min of incubation and the samples were centrifuged at the desired pH at 50 ml h⁻¹. The supernatant was discarded; the proteic suspension was 1 mg/ml. The reaction was stopped after 15 min of incubation and the samples were centrifuged at the desired pH at 50 ml h⁻¹. The supernatant was discarded; the proteic suspension was 1 mg/ml. The reaction was stopped after 15 min of incubation and the samples were centrifuged at the desired pH at 50 ml h⁻¹. The supernatant was discarded; the proteic suspension was 1 mg/ml. The reaction was stopped after 15 min of incubation and the samples were centrifuged at the desired pH at 50 ml h⁻¹. The supernatant was discarded; the proteic suspension was 1 mg/ml. The reaction was stopped after 15 min of incubation and the samples were centrifuged at the desired pH at 50 ml h⁻¹. The supernatant was discarded; the proteic suspension was 1 mg/ml.

**Time-resolved Fluorescence Measurements**

Fluorescence intensity and anisotropy decays were obtained by the time-correlated single photon counting technique from the polarized components Iₓ(t) and Iᵧ(t) on the experimental set-up installed on the SB1 window of the synchrotron radiation machine Super-ACO (Anneau de Collision d’Orsay) (34). Time resolution was ~10 or 20 ps, and the data were stored in 2048 channels. Automatic sampling cycles including 50 s of accumulation time for the instrument response function and 90 s of acquisition time for each polarized component were carried out such that a total number of 2–4 × 10⁶ counts was reached in the fluorescence intensity decay. Analyses of fluorescence intensity and anisotropy decays as sums of exponentials were performed by the maximum entropy method (MEM) (35–37).

**Curve Fitting**

Aggregation and binding curves were obtained by fitting the experimental data to the equation y = a + (b − a)(1 + (t − a) / b)⁰⁻ᵃ in which a is the minimal y; b is a constant; and n a constant representing the Hill number. The statistical analysis and curves fitting were performed with Graphpad Prism software.
RESULTS

pH-dependent Binding of AnxA2 and (AnxA2-P11)_2 to Liposomes—Preliminary to the study of annexin 2 binding to liposomes at acidic pH, it was verified that AnxA2 and (AnxA2-P11)_2 remained soluble at the pH used for these experiments (from pH 7 to 4). The dissociation of (AnxA2-P11)_2 was also evaluated. For this purpose, (AnxA2-P11)_2 was subjected to exclusion chromatography at different pH values. These experiments demonstrated that the tetramer is 100% stable at pH from 7.4 to 6.5. At pH 6, P11 started to dissociate, and 60% of the protein remained in a tetrameric form. However, aggregation and binding experiments showed that, at pH 6, a condition in which the monomeric form is not bound to liposomes, the aggregation and binding of the tetrameric annexin 2 was in the range of 60–90% (see below). We concluded that at pH 6, 60–90% of the protein remained in the tetrameric form. Because the small fraction of monomeric AnxA2 at pH 6 is inactive (not bound), we were able to work properly with the tetramer in the pH 7–6 range.

The binding of AnxA2 and (AnxA2-P11)_2 to PC/PS unilamellar liposomes was monitored at different pH values. Liposome binding of annexin 2 increased with the H^+ concentration (Fig. 1A). The binding of (AnxA2-P11)_2, was detected at pH just below neutrality and showed 50% of bound protein at pH 6.3. The binding of AnxA2 started at a higher H^+ concentration (pH lower than 6) and exhibited a half-maximal value at pH 5.7. It is worth noting that the binding efficiency of AnxA2 was more than 90% at pH 5.

The binding of P11 dimer to PC/PS liposomes was also studied (Fig. 1A). Unexpectedly, P11 bound to liposomes at acidic pH. Although the slopes of binding for (AnxA2-P11)_2 and P11 (Hill number ~1.24 and ~0.59, respectively) were different, as well as the pH range of binding (pH 7–6 and pH 6–4, respectively), it remained unclear whether the small fraction of membrane-bound P11 observed at pH 6 interferes with the binding to membranes of (AnxA2-P11)_2 and, if so, to what extent.

Because EGTA is a poor chelating reagent at acidic pH, the involvement of Ca^{2+} at acidic pH was investigated in the presence of EGTA (10 mM), a chelating agent with higher affinity for Ca^{2+}. Fig. 1B shows that at pH 4.5 in the presence of EGTA half of the protein is associated to membranes. If EGTA is a good chelator of Ca^{2+} at this pH, then the membrane-bound protein does not result from Ca^{2+}-mediated binding. If EGTA is not an efficient chelator and if Ca^{2+} is involved in binding, then the addition of 1 mM Ca^{2+} must increase binding. Fig. 1B shows that this is not the case; moreover, in the presence of 1 mM Ca^{2+} and in the absence of EGTA the fraction of protein associated to membranes is practically identical. These experiments show that Ca^{2+} is not involved in the acidically mediated membrane binding of AnxA2.

Requirement of Negatively Charged Phospholipids for the Binding of AnxA2 to Liposomes at Acidic pH—AnxA2 was added to PC and to PC/PS liposomes at pH 4.7 (Fig. 1C). After centrifugation, no protein was detected in the pellets from pure PC liposomes, indicating that AnxA2 was not able to bind to PC liposomes at acidic pH. The binding of AnxA2 to liposomes required the presence of PS. AnxA2 was also able to associate with liposomes containing other negatively charged lipids including phosphatidylglycerol and phosphatidylinositol (data not shown).

Influence of the Ionic Strength on Binding and Release of AnxA2 at Acidic pH—The effect of ionic strength on the pH-induced binding of AnxA2 was investigated. AnxA2 binding at pH 5.2 and 4.5 was studied in the presence of increasing KCl concentrations. The binding of AnxA2 to PC/PS liposomes was inhibited by KCl (Fig. 1D). At both pH values, the decrease in binding started at 40 mM KCl with a 50% inhibition obtained at 45 mM and 50 mM KCl for pH 5.2 and pH 4.5, respectively. Thus, ionic force reduced the binding of AnxA2 to negatively charged phospholipids, and this inhibition was less efficient at high H^+ concentration. Because ionic strength hindered the binding of AnxA2, we studied whether AnxA2 previously bound to liposomes could be released by an increase in ionic strength. AnxA2 was first incubated with liposomes at pH 5.2 in the presence of 30 mM KCl, and then the annexin-liposome complexes were incubated with either 100 or 200 mM KCl. Under both conditions, the protein was dissociated from membranes (70% solubilization, Fig. 1E). These results are consistent with an electrostatic nature of the pH-mediated binding.

pH-dependent Membrane Aggregation Mediated by AnxA2 and (AnxA2-P11)_2—Membrane aggregation properties of AnxA2 and (AnxA2-P11)_2 were also investigated at acidic pH in the absence of Ca^{2+}. Both forms induced PC/PS liposome aggregation, although the extent of aggregation depended on the nature of the annexin form and occurred at different pH values (Fig. 2). (AnxA2-P11)_2 aggregation started at a pH lower than 6.5 and reached a value of 60% at pH 6. In the case of AnxA2, the aggregation was observed at a lower pH (below pH 5.5) with a half-maximal value at pH 4.7.

The pH-mediated aggregation properties of (AnxA2-P11)_2 were also studied on biological membranes. (AnxA2-P11)_2 started to aggregate chromaffin granules below pH 7 and attained 90% aggregation at pH 6 with an apparent half-maximal aggregation at pH 6.3, exhibiting more efficient aggregation than with liposomes. Chromaffin granules started to precipitate at pH 5 precluding the study of aggregation by AnxA2.

Because (AnxA2-P11)_2-mediated chromaffin granules aggregation showed higher H^+ sensitivity than the PC/PS liposomes, we examined the possibility that the lipid composition was involved in this change on H^+ sensitivity. Because the presence of cholesterol in PC/PS liposomes has an influence in the Ca^{2+}-dependent and Ca^{2+}-independent binding and aggregation of annexins (19, 23), we characterized the membrane aggregation of annexin 2 with PC/PS/cholesterol (50:25:25) liposomes. As shown in Fig. 2, the H^+ sensitivity of aggregation of cholesterol-containing liposomes was increased for both forms of annexin 2. The half-maximal aggregation value for AnxA2 was shifted from 4.7 in PC/PS liposomes to 5.6 in PC/PS/cholesterol liposomes. With the tetramer, the H^+ sensitivity was also shifted with 80% of aggregation at pH 6. These experiments suggest that, similarly to the Ca^{2+}-mediated properties, the lipid composition and/or the formation of membrane microdomains is important for the H^+ mediated binding and aggregation. The interactions of annexin 2 with membranes at acidic pH in the absence of Ca^{2+} were investigated in more detail.

Observation by Cryo-TEM of Liposomes Aggregated by AnxA2 at Acidic pH—Liposome aggregates induced by annexin 2 at acidic pH were subjected to cryo-TEM observation. Within these aggregates, a peculiar organization of electron dense material was visible between the lipid bilayers of two juxtaposed liposomes exhibiting a so-called junction. This dense material was arranged into either one or two layers between the membranes (Fig. 3). Each lipid bilayer was resolved into two dark stripes corresponding to the lipid polar head groups. When one extra layer was present, the junction exhibited five dark stripes with an overall thickness of about 135 Å, which were interpreted as, successively, two lipid leaflets of the first membrane, one layer of AnxA2 molecules, and two leaflets of the second lipid membrane (Fig. 3, A–C). When two protein layers were present, the junction exhibited six dark stripes with an overall thickness of about 165 Å proposed to contain.
successively, two leaflets of one lipid membrane, two layers of AnxA2 molecules, and two lipid leaflets of the other membrane (Fig. 3, D–F).

Although lipidosome aggregation was mediated through a direct contact between two liposomes, it is important to note that another type of contact was frequently observed. One liposome could be partially or totally encapsulated into a larger liposome revealing invaginated assemblies (Fig. 3, G and H) and multilamellar structures (not shown). The contact between these two liposomes appeared as curvilinear junctions made of
two lipid bilayers and one or two layers of AnxA2 molecules. The features of these curved junctions strongly suggested that AnxA2 molecules would cover the whole surface of the encapsulated liposome. Because such peculiar assemblies did not exist with pure liposomes, it was evident that AnxA2 induced the formation of encapsulated liposomes.

Aggregation of (AnxA2-P11)2-Liposomes Observed by Cryo-TEM at Acidic pH—Liposome aggregates obtained with (AnxA2-P11)2 at pH 6 were subjected to cryo-TEM (Fig. 4). Electron dense material was observed between the liposome membranes forming junctions (Fig. 4, A, C, and D). The arrangement of (AnxA2-P11)2 between the liposomes looked fuzzy. Unlike AnxA2, (AnxA2-P11)2 did not produce regular stripes, revealing that the tetramer complexes were not arranged in a regular motif. Although the thickness of the junctions seemed rather constant (165 Å), the fine structure of these junctions did not reveal regular stripes that could be assigned to the AnxA2 and P11 (Fig. 4, C and D). The organization of (AnxA2-P11)2 at low pH contrasted with those at neutral pH in the presence of Ca2+ (Fig. 4, B and E). In the latter case, the junctions were formed by three dark stripes between the lipid bilayer, which were assigned to an AnxA2 sequence (38).

It must be noted that, at acidic pH, curved junctions were observed quite often. Like AnxA2, (AnxA2-P11)2 induced the formation of invaginated liposomes. Because such peculiar assemblies did not exist with pure liposomes, it was evident that AnxA2 induced the formation of encapsulated liposomes.

Reversibility by pH Neutralization of the Binding of AnxA2 to Liposomes—The reversibility of the pH-induced AnxA2 membrane binding was studied by formation of liposomes-AnxA2 complexes at pH 4 and then incubation of these complexes at neutral pH. In these conditions, the liposome-AnxA2 complex dissociated only partially (70%) (Fig. 5A). The reversibility of junction formation was also studied by cryo-TEM. AnxA2 was mixed with liposomes at pH 4, and then the pH of the sample was increased up to pH 7. Fig. 5B shows the persistence of multilamellar structures containing AnxA2 molecules. This suggested that the part of AnxA2, which was not releasable at neutral pH, likely corresponds to encapsulated protein in the multilamellar structures.

pH-induced Conformational Changes of AnxA2 Studied by Time-resolved Fluorescence—It has been proposed that at acidic pH in the presence of phospholipid membranes, annexins 12 and 5 adopt a transmembrane conformation (28, 29). However, the fact that the membrane aggregation property of annexin 2 as well as the proteic bridges were present under mild acidic conditions suggested that the overall structure of at least repeats I and IV known to be involved in membrane aggregation were conserved (39, 40).

In order to check for possible pH-induced conformational changes, the fluorescence properties of the single tryptophan, Trp-212, present in the repeat III of the wild type protein and those of a mutant protein containing a single tryptophan in repeat II (L127W/W212F) were studied. The emission spectra of both proteins were recorded at pH 7, pH 4, and pH 4.5 in the presence of PC/PS liposomes. The emission spectrum of L127W was identical in all conditions with an emission maximum at 336 nm indicating a high stability of repeat II. On the contrary, the Trp-212 of repeat III showed differences in the fluorescence emissions, suggesting that the part of AnxA2, which was not releasable at neutral pH, likely corresponds to encapsulated protein in the multilamellar structures.

The Trp-212 fluorescence intensity decay in AnxA2 solubilized in pH 4 buffer was heterogeneous. Three lifetime populations characterized it, the longest lived one largely dominated (Fig. 6). This lifetime distribution was very close to those measured at neutral pH in the presence of Ca2+, suggesting a similar local conformation in both conditions (16).

In the presence of negatively charged membranes at pH 4.5, the fluorescence decay of Trp-212 in AnxA2 was very similar to that in the absence of membranes (Fig. 6). Therefore, the conformation of repeat III changes in solution at mild acidic pH, but it does not undergo any additional change upon binding to
membranes. The Trp-212 environment of AnxA2 at pH 4.5 resembled its Ca\(^{2+}\)-bound form (16). In this respect, the pH-switch of the conformational transition of repeat III seems to be equivalent to the Ca\(^{2+}\) switch.

To monitor the Trp-212 mobility, anisotropy decay measurements were also performed. The MEM analysis of the polarized fluorescence decays of Trp-212 in AnxA2 showed the presence of a single long rotational correlation time distribution describing the Brownian rotation of the whole protein (26 ns, \(\theta\) in Table I). This correlation time is shorter than that observed at pH 7 in the absence of Ca\(^{2+}\) (34 ns) but close to the one observed at pH 7 in the presence of Ca\(^{2+}\) (28 ns) (16). Thus, the protein seemed to be more compact at pH 7 in the presence of Ca\(^{2+}\) as well as at pH 4.5. The initial anisotropy value \((A_{i=0} = 0.18)\) is lower than the value of 0.25 expected for the immobilized Trp-212 from previous measurements (16), indicating that faster rotations may exist. The amplitude of the Trp-212 local rotational motion (26\(^{\circ}\) as estimated from the value of the semi-angle of the wobbling-in-cone sub-nanosecond motion \(\omega_{\text{max}}\)) was more restricted at pH 4.5 than at pH 7 in the absence of Ca\(^{2+}\) (33\(^{\circ}\)), but was similar to those at pH 7 in the presence of Ca\(^{2+}\) (25\(^{\circ}\)) (16).

The binding of AnxA2 to the negatively charged membranes induced a large increase in the long rotational correlation time to a much longer value (54 ns), almost infinite with respect to the time scale of the fluorescence intensity decay (Table I). This rotational time was correlated with the protein-liposome complex.

However, the amplitude of the local motion of Trp-212 did not significantly change as compared with that occurring in the absence of membranes \((\omega_{\text{max}}\) in Table I), underlying that neither additional conformational nor dynamic changes were brought about by binding of the protein to the membranes.

**Influence of the pH on the Stokes Radius of AnxA2**—The decrease in both the thickness of membrane junctions observed at acidic pH compared with those at neutral pH in the presence of Ca\(^{2+}\), and the decrease in fluorescence correlation time in the presence of H\(^+\) and Ca\(^{2+}\) suggested that the protein would be more compact at acidic pH. Thus, the Stokes radius of AnxA2 at different pH values was studied. As shown in Fig. 7, the Stokes radius of the protein was 2.2 ± 0.01 nm, a value which is very close to the value reported by Gerke and Weber (1). This value was stable at pH 7, 6.5, and 6 but decreased at pH 5.5 and 5 to a value of 1.83 ± 0.04 nm. This diminution could result from a compaction, a reduction in flexibility, or dehydration of the protein. As H\(^+\) and Ca\(^{2+}\) seemed to share some properties, we measured the Stokes radius of AnxA2 at pH 7 in the presence of 0.1 mM Ca\(^{2+}\). The experiment gave a value of 1.74, which is close to what is observed at pH 5.5 and less.

**Protein-Membrane Interactions Observed by \(^{125}\)TID Labeling**—To further study the interaction of AnxA2 with membranes, we compared the labeling of AnxA5 and AnxA2 with the lipophilic probe \(^{125}\)TID. This photoactivatable reagent partitions into lipid bilayers and labels membrane proteins. By using this probe, Issas et al. (29) suggested that, at acidic pH and thanks to a dramatic conformational change, AnxA5 and AnxB12 insert into the lipid bilayer.

In a first series of experiments, we used the protocol described previously (29), in which the protein was incubated with the liposomes, and after that the radioactive probe was added. In those conditions we observed labeling of AnxA5 and AnxA2 at acidic pH but, unexpectedly, also labeling incorporation at neutral pH in the presence of Ca\(^{2+}\). To discard the possibility that these labelings were due to association of the probe with hydrophobic regions of the protein which became accessible during the formation of protein-protein interactions at the membrane surface, we changed the protocol. First, we incubated the liposomes with the probe; second, we added the protein to the probe-containing membranes.

As shown in Fig. 8, AnxA5 was strongly labeled at pH 4.5 in the presence of liposomes (even if only a fraction of the protein is labeled, this signal was arbitrarily designed as 100%); AnxA5 was slightly labeled at pH 7 in the presence of membranes and Ca\(^{2+}\) (~20%) and at pH 4.5 in the absence of membranes (~40%). The behavior of AnxA2 at pH 4.5 was similar to that observed for AnxA5. AnxA2 showed weak labeling at pH 4.5 in solution, and as in the first experiments, it was strongly labeled in the presence of membranes at pH 4.5 (as for annexin 5 a

**TABLE I**

| Sample                  | \(\theta\) (ns) | \(\Sigma \beta_i = A_{i=0}\) | \(\omega_{\text{max}}\) (*) |
|-------------------------|-----------------|-----------------------------|-----------------------------|
| AnxA2                   | 26              | 0.180                       | 0.180                       |
| AnxA2 + PC/PS liposomes | 54              | 0.180                       | 0.180                       |

The fluorescence anisotropy is assumed to be described by a sum of exponentials: 
\[ A(t) = \left( I_{\text{in}}(t) - \beta_{\text{corr}} I_{\text{in}}(0)/I_{\text{in}}(t) + 2\beta_{\text{corr}} I_{\text{in}}(t) = \Sigma \beta_i \exp(-t/\tau_i) \right) \] 
where \(A_0 = \Sigma \beta_i \) ; \(\tau_{\text{corr}}\) is the correction factor. Excitation wavelength, 295 nm; emission wavelength, 320 nm. The \(\theta\) and \(\beta\) parameters are, respectively, the values of the center and of the partial anisotropy corresponding to each rotational correlation time peak. The wobbling-in-cone angle \(\omega_{\text{max}}\) was calculated as follows: 
\[ \beta P_o (1/2 \cos \omega_{\text{max}} (1 + \cos \omega_{\text{max}})^2) \] 
A value of the intrinsic anisotropy \(A_0\) of 0.25 was used.

**FIG. 7.** Stokes radius of AnxA2 as a function of pH. The Stokes radius was obtained as described under “Experimental Procedures.” (\(n = 1–5\) measurements.)
value of 100% was assigned). However, the presence of Ca\(^{2+}\) does not modify substantially the labeling of the protein at acidic pH. At pH 7 plus 1 mM free Ca\(^{2+}\), the AnxA2 labeling was stronger (~140%). Both annexins were very weakly labeled at pH 7 in the absence of membranes (10–15%). These results indicate first that at acidic pH in solution, both annexins increased their hydrophobicity and are slightly labeled. Second, AnxA5 labels strongly in the presence of membranes only at acidic pH. On the contrary, the interaction with membranes provokes the labeling of AnxA2 at both acidic and neutral pH in the presence of Ca\(^{2+}\). Notice that in the last condition, the protein does not change its global structure. Thus, we conclude that there is no need of a dramatic conformational change for AnxA2 to be labeled by TID.

Labeling experiments were also performed with the tetrameric protein at pH 7. The results showed that the AnxA2 in the (AnxA2-P11)\(_2\) complex is able to be labeled at pH 7 in the presence of membranes and Ca\(^{2+}\) and only weakly in the absence of membranes and/or Ca\(^{2+}\) (not shown).

Characterization of TID-labeled Domains after Interaction of AnxA2 with Membranes—To characterize the zones of the protein in contact with the hydrophobic portion of the membrane bilayer, we performed several analyses by mass spectrometry on tryptic peptides. Peptide masses were measured as described under “Experimental Procedures.” We compared the masses of peptides derived from unlabeled AnxA2, with peptides from AnxA2 labeled in solution or associated with membranes at acidic pH or at neutral pH in the presence of Ca\(^{2+}\) (Table II), and we searched for peptides incremented by 284.021 daltons, the mass that corresponds to the addition of TID-peptide product.

Three regions were found labeled by the \(^{125}\)I-TID. The peptide 304–309 was found in all conditions, and the peptide 295–307 was observed at pH 4.5 in the presence of membranes (Table II). These peptides overlap in the region 304–307, which indicates that the SEFK residues in the helix C of repeat IV (Fig. 9) could be the TID target. A second peptide 80–87 (KELASALK) was also found in all conditions (Table II), and this peptide corresponds to parts of the third loop and of helix D of repeat I, and its last Lys is very close to one of the Ca\(^{2+}\)-ions bound to repeat I (Fig. 9). The third labeled region was observed for the protein associated with membranes both at pH 4.5 and at neutral pH in the presence of Ca\(^{2+}\). This “membrane-specific” labeled region corresponds to residues 196–204 (Table II). These residues (DLYDAGVKR) form part of the first helix (helix A) of the third repeat and the first part of the loop between helices A and B (Fig. 9). Notice that the last Lys and Arg of this sequence are very close to the Ca\(^{2+}\)-binding site and thus to the membrane.

DISCUSSION

The mechanism of Ca\(^{2+}\)-dependent phospholipid binding of annexins implies the interaction between the lipid head groups and the convex face of the annexin core. Recently, it has been reported that annexins can bind to membranes in a Ca\(^{2+}\)-independent manner under neutral (18–22) and acidic conditions (28, 29). In this paper, the binding and aggregation of annexin 2 at acidic pH without Ca\(^{2+}\) have been investigated.

Is Annexin 2 a Transmembrane Protein at Acidic pH?—Two hypotheses can be proposed to account for the observed properties of annexin 2 binding to membranes at acidic pH: the “conformational transition” or the “electrostatic hypothesis.” The first one is based on experiments with AnxB12 (28) in which 24 spin-tagged residues (extending from the D helix of repeat II to the loop connecting repeats II and III) showed a sequence of EPR spectra similar to that corresponding to an amphipathic transmembrane helix. Therefore, the protein was proposed to adopt a transmembrane conformation (Fig. 10B). This hypothesis has been extended to AnxA5. The insertion of a transmembrane segment was also suggested by electrophysiological data that showed much more channel activity at acidic than at neutral pH (29).

Specific experiments were performed to test this hypothesis for annexin 2. (i) To search for a possible restructuring of
Ca\(^{2+}\) - and pH-mediated Annexin 2-Membrane Interactions

### MALDI-TOF MS results obtained after reduction-alkylation and trypsin digestion of annexin 2 in reflector mode

| Measured MH\(^{+}\) | Theoretical MH\(^{+}\) | Δ mass | Sequence | Position | TID-modified MH\(^{+}\) (measure) |
|---------------------|---------------------|--------|----------|----------|----------------------------------|
| 536.296             | 536.283             | 24     | VFDR     | 227–230  |
| 575.281             | 575.290             | 7      | SNAQR    | 63–67    |
| 666.355             | 666.357             | 2      | SEFRKR   | 304–308  |
| 756.353             | 756.360             | 9      | LYDSMK   | 273–278  |
| 779.459             | 779.441             | 23     | IRSEFK   | 302–307  |
| 808.432             | 808.468             | 45     | SVPHLKQ  | 220–226  |
| 794.452             | 850.023             |        | SEFRKR   | 304–309  |
| 880.439             | 880.441             | 2      | DLYDAGVK | 196–203  |
| 1035.529            | 1035.5292           | 0      | WISIMTER | 212–219  |
| 1096.489            | 1096.485            | –12    | AYNFDAER | 29–36    |
| 1111.574            | 1111.553            | –19    | QDIAFAYQ | 68–76    |
| 1128.556            | 1128.5718           | 14     | STVHEILC | 1–9      |
| 1222.575            | 1222.595            | 16     | TPAQYDASELK | 104–114 |
| 1244.654            | 1244.6229           | –25    | TNQELQEINR | 135–144 |
| 1353.719            | 1353.664            | 41     | DIISDTSGDFRK | 157–168 |
| 1421.708            | 1421.6947           | –9     | SLYYYIQQDTK | 313–323 |
| 1460.701            | 1460.6726           | –19    | SYSPYDMLESIR | 233–244 |
| 1542.572            | 1542.8485           | –15    | GVDVETIVNILTNR | 49–62    |
| 1588.814            | 1588.768            | 29     | SEVDMLKIRSEFK | 295–307  |
| 1650.993            | 1650.978            | –8     | SALSGLTVLIGLLK | 88–103   |
| 1720.866            | 1720.8421           | –14    | GLGTIEDSLIEICRS | 119–134  |
| 1777.893            | 1777.8636           | –17    | GLGTIEDSLIEICRS | 119–134  |
| 1811.907            | 1811.8657           | –23    | TDLEEIDSGDGFKR | 152–167  |
| 1844.919            | 1844.9024           | –9     | LSLQDIHTTPPSAYGQK | 10–27   |
| 1808.915            | 1908.8821           | –17    | AEDGSVDYELIDQDAR | 179–195  |
| 1939.974            | 1939.9607           | –7     | TDLEEIDSGDGFKR | 152–168  |
| 2064.985            | 2064.9832           | –1     | RAEDGSVDYELIDQDAR | 178–195  |
| 2155.087            | 2155.0685           | 0      | AYNFDAERDALNIEATAK | 29–46    |

* TID-modified peptides were measured at pH 4.5 in the presence of membranes.
* TID-modified peptides were measured at pH 4 in the presence of membranes and Ca\(^{2+}\).
* TID-modified peptides were measured at pH 4 in solution.

AnxA2 after membrane binding, the fluorescence properties of the Trp-212 of repeat III and of the mutant L127W/W212F of repeat II were analyzed. At acidic pH, the fluorescence emission spectra did not change when the protein was associated to membranes. Our fluorescence data are consistent with the work of Rosengarth et al. (41) who showed that the overall secondary structure of AnxA1 was not affected by large variations in pH (3.8–8.0). On the other hand, repeats I and IV have been proposed to be involved in intermolecular interactions during membrane aggregation of annexins 1 and 2 (39, 40). The fact that AnxA2 was competent for aggregation at acidic pH indicated that the structure of the two repeats was preserved. (ii) When annexin 2 in its monomeric and tetrameric forms was indicated that the structure of the two repeats was conserved.

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Fig. 9. \(^{125}\)I-TID-labeled regions of AnxA2. The three-dimensional structure of AnxA2 is represented in lateral (A) and “rear” (B) views. The membrane-binding convex face is at the bottom. The Ca\(^{2+}\) ions are depicted as dark gray round clouds. The residues 304–307 in repeat IV and 80–87 in repeat I that were labeled in all conditions are represented by gray ribbons. The helix-loop region (196–204) of repeat III, which was labeled only in the presence of membranes both, at acidic pH and at pH 7 in the presence of Ca\(^{2+}\), is depicted with the black ribbon.

M. Thieffry and J. P. Henry, unpublished observations.
labeled at acidic pH in the presence of membranes. This result is similar to that obtained by Isas et al. (29). However, we observed that in solution AnxA5 was labeled at pH 4.5 but not at pH 7, indicating an increase in the hydrophobicity of the protein at acidic pH in the absence of membranes (not shown). For AnxA2, we showed that the protein was labeled in two regions (80–87 and 304–307) in the absence and the presence of membranes, suggesting that these regions possess an intrinsic affinity for the probe in all conditions. It was also observed that in the presence of membranes at both acidic and neutral pH with Ca\(^{2+}\), the protein was labeled in a quite specific region (helix A and the first loop of repeat III). At pH 7 in the presence of Ca\(^{2+}\), the protein is known to conserve its overall three-dimensional structure. Then, the labeling of protein with TID cannot be unambiguously interpreted for the purpose of discerning transmembrane protein domains. This problem has been described for other proteins such as the cytosolic domains of the H\(^{+}\)-ATPase (42) and the active site of Cox (43). Thus, caution should be applied in the interpretation of TID-labeling patterns. On the other hand, if AnxA2 changed its conformation at pH 7 (Fig. 10B), then we expected to observe more TID-labeled peptides in different regions of the protein. This was not observed. Therefore, no evidence for a large conformational change specifically mediated by membrane binding at acidic pH could be found.

In the second hypothesis, AnxA2 would bind to membranes at low pH in a manner analogous to the Ca\(^{2+}\)-mediated binding, i.e. by electrostatic interactions between positively charged amino acid residues and negatively charged phospholipid head groups (Fig. 10A). In this case, the protein must conserve its native three-dimensional structure.

All the data presented here exclude the hypothesis of the insertion of one or more transmembrane segments (Fig. 10, B and C) and support the hypothesis of a Ca\(^{2+}\)/H\(^{+}\) binding, facilitated at the membrane/protein interface (Fig. 10A).

**Similarities between Ca\(^{2+}\)- and H\(^{+}\)-mediated Annexin 2-Membrane Interactions**—This study revealed similarities between Ca\(^{2+}\)- and H\(^{+}\)-mediated AnxA2-membrane interactions. First, AnxA2 and (AnxA2-P11)\(_2\) were able to bind to and to aggregate membranes at mild acidic pH. These results imply that the core domain of the protein is responsible for H\(^{+}\)-mediated membrane binding. Second, binding and aggregation were observed at similar Ca\(^{2+}\) or H\(^{+}\) concentrations. Half-maximal PC/PS liposome binding for AnxA2 was observed at pH 5.7 or pCa 5.7 and for (AnxA2-P11)\(_2\) near pH 6.4 or pCa 6.1. Half-maximal aggregation for AnxA2 was observed at pH 4.7 or pCa 4 and for (AnxA2-P11)\(_2\) near pH 6.2 or pCa 6.5 (17). In terms of efficiency, it should be noted that the maximal aggregation of liposomes (for AnxA2) or chromaffin granules (for (AnxA2-P11)\(_2\)) induced by acidic pH occurs in the same range as that obtained in the presence of Ca\(^{2+}\) (17, 19). Third, Ca\(^{2+}\)- and H\(^{+}\)-mediated membrane interactions required the presence of negatively charged phospholipids. This requirement of anionic phospholipids for binding at acidic pH was also observed for AnxA5 and AnxB12 (26, 29). Fourth, the presence of cholesterol in the liposomes increased the H\(^{+}\) sensitivity for aggregation. Fifth, an increase of the ionic strength of the solution induced a dissociation of AnxA2 binding at low pH as in the presence of Ca\(^{2+}\) (44). A sixth argument deals with the fluorescence properties of Trp-212. At acidic pH, repeat III exhibited a conformational change almost identical to that observed in the presence of Ca\(^{2+}\) at neutral pH (16). The interaction of AnxA2 with negatively charged membranes at acidic pH or at neutral pH in the presence of Ca\(^{2+}\) did not result in any further conformational change of repeat III. Finally, the protein was labeled by the TID in the same region of repeat III at acidic pH as well as a neutral pH in the presence of Ca\(^{2+}\) revealing similar interaction with the membrane in both conditions.

**Mechanism of Binding**—Our experiments indicate that Ca\(^{2+}\) is not involved in the H\(^{+}\)-mediated membrane binding of annexin 2. Moreover, the glutamic and aspartic acid residues of the Ca\(^{2+}\)-binding sites of AnxA2 are probably protonated at acidic pH, a fact that would diminish the affinity for Ca\(^{2+}\). The results obtained in mild acidic conditions favor a mechanism involving electrostatic interactions between annexin 2 and phospholipids. These interactions might occur between lysine side chains protruding the convex face of the four repeats of the protein and the negatively charged phospholipid head groups. In this regard, it is important to note that residues outside the Ca\(^{2+}\)-binding sites could stabilize the interaction of the protein with membrane phospholipids (45, 46). Recently, Montaville et al. (47) proposed the existence of a PS-binding site in the repeat I of AnxA5. The oxygen atoms of the PS head group could be able to form hydrogen bonds with residues Arg-25, Lys-29, and Arg-63. These residues are conserved among annexins and correspond to Lys-115, Lys-119, and Lys-152 in repeat II of AnxA2. Decreasing the pH would partially protonate the acidic side chains involved in the Ca\(^{2+}\)-binding sites resulting in the reorientation of the neighboring basic residues involved in the PS-binding site and allowing the formation of hydrogen bonds with the PS head.

The TID experiments indicate that the protein interactions with the hydrophobic part of the bilayer are close enough to label the protein. Other soluble proteins such as PC-cytidyltransferase (48), cytosolic phospholipase A2 (49), proteinase 3 (50), and apolipoprotein A-I (51), which are able to associate to membranes, have been described to be labeled by TID in domains supposed to insert only partially and reversibly into the membranes. In the case of AnxA2, the labeling at both pH 4.5 and pH 7 in the presence of Ca\(^{2+}\) could be due to a quite superficial insertion of the repeat III (“snorkeling”) thanks to an increase in protein accessibility to the hydrophobic part of the membrane by spreading the polar head groups of bound phospholipids. Probably the best target groups in proteins for the reactive carbene in TID are the OH and NH groups, suggesting that the residues close to the membrane, Lys-203 and Arg-204 in the first loop of repeat III, are the best candidates to be TID targets.

**Specificity of H\(^{+}\)-mediated Membrane Junctions Organization**—The main differences between H\(^{+}\)- and Ca\(^{2+}\)-mediated protein-membrane interaction concerned the membrane junctions. The organization of the junctions at acidic pH was quite different from that observed at pH 7 in the presence of Ca\(^{2+}\), conditions in which (AnxA2-P11)\(_2\) and AnxA2 are organized between the membranes in three and two protein layers, respectively (38).

AnxA2—The monomer presented two types of junctions: a five stripe junction of 135 Å containing one protein layer and another with two proteic layers observed less frequently with a thickness quite similar to that of the Ca\(^{2+}\)-mediated junction (165 Å). The difference between these two types of junctions (about 30 Å) is close to the AnxA2 thickness (mean of 36 Å between the concave and convex faces). The dehydration and/or compaction of the protein measured by the Stokes radius that we observed at pH 5 in solution (our exclusion chromatography and fluorescence correlation time experiments) cannot explain satisfactorily the diminution of the membrane thickness because this decrease in the Stokes radius was also observed for the protein at pH 7 in the presence of Ca\(^{2+}\). We propose then that the determinant factor in the junction morphology and properties is the difference in the organization of the annexin.
Ca\(^{2+}\) - and pH-mediated Annexin 2-Membrane Interactions

**Fig. 11. Models for the organization of annexin 2 junctions at mild acidic pH.**

A. AnxA2 binds two membranes by the concave and the convex faces of its core. B, AnxA2 binds one membrane by the convex face and the other membrane by its N-terminal tail. C, the annexin cores of the tetramer bind each one on an opposite membrane by their convex faces. The tetramer cores are shifted (hinge effect). D, the annexin cores of the tetramer bind the same membrane. The aggregation of membranes is mediated by contacts of the concave faces of opposite cores.

For the less represented six stripes junction, the protein could be organized face-to-face as suggested for the Ca\(^{2+}\)-mediated junctions (38). For the five stripes junction, the protein could interact with both membranes as proposed for AnxA1 (52). In this model, the protein binds one membrane by the convex face and the other membrane could be bound by a secondary binding site as suggested for AnxA1 (53) either by its concave face (Fig. 11A) or by the N-terminal domain (Fig. 11B).

In both possibilities, the proteic complexes could be organized either in an asymmetrical network in which all the proteins had its convex face bound to one membrane and the concave face facing the other membrane, or in a symmetrical structure with proteins in both orientations (Fig. 11, A and B).

AnxA2-P11 (AnxA2-P11)-The junction induced at pH 7.4 in the presence of Ca\(^{2+}\) exhibited three layers of protein corresponding to an AnxA2 layer, a P11 dimer layer and an AnxA2 layer, respectively (38). The thickness of the tetramer junction was decreased from 210 Å at pH 7.4 in the presence of Ca\(^{2+}\) to 165 Å at acidic pH, indicating a different organization of the tetramer. The junction induced at acidic pH exhibited electron densities that were not distributed in regular stripes. Although these densities were likely attributed to (AnxA2-P11), the organization of the complex remains unclear, and no particular density could be assigned to AnxA2 or to P11. The 45-Å decrease in the mean junction thickness could be interpreted as the loss of a protein in the complex. However, the differences in the behavior of the monomer and the tetramer as well as our biochemical studies indicate that this is not the case.

Two possibilities for the organization of the tetramer at acidic pH are considered. The first possibility is a tetrameric complex in which the AnxA2-(P11)\(_{2}\)-AnxA2 is proposed to be a hinge. In this case, the annexin molecules could move around P11 allowing the formation of a junction with the annexin cores binding the membranes by the convex faces but shifted around the P11 axis (Fig. 11C). The second possibility is an open AnxA2-(P11)\(_{2}\)-AnxA2 complex in which one membrane binds both convex faces of the tetramer (Fig. 11D). There the junction could be obtained by the interaction of two opposite annexin cores. These possibilities imply the movement of the two cores around P11, at variance with the structure proposed for the Ca\(^{2+}\)-induced junctions.

Another important property observed was the curvilinear shape of the AnxA2 and (AnxA2-P11)\(_{2}\) junctions formed at acidic pH, with images of invaginations and the formation of multilamellar liposomes. These structures were not observed in the presence of Ca\(^{2+}\), where the junctions are planar, highly symmetrical, and apparently in which the AnxA2 molecules are well organized face-to-face.

Arginine side chains are mostly present on the concave face of the protein and could be involved in a second type of contact with the opposite membrane. Overall, the distribution of the electrostatic surface potential of the AnxA2 molecule shows a strong positive region in its convex face and in the lateral side of repeat I. Conversely, repeat III displays a more negative surface potential at moderately acidic pH. It could participate, however, in lateral contacts with neighboring repeats I. The protein-membrane assemblies formed at acidic pH could therefore display a different topology than classically formed at neutral pH in the presence of Ca\(^{2+}\). The orientation of the protein at the membrane surface in mild acidic pH conditions might be less ordered than the classical one at neutral pH, which is maintained by the Ca\(^{2+}\) bridges (54). It might be proposed that the curvilinear junctions comprise tetramers bound in the two modes described in Fig. 11, C and D; in the tetramer, annexin cores in a cis-disposition (Fig. 11D) would not bridge the two membranes; they would be more abundant on the convex side of the junction, acting as spacers and thus increasing its curvature. To allow the junctions, annexin 2 cores in the trans-disposition (Fig. 11C) would be obtained by a rotation around the hinge. Such a hypothesis would account for the irregular fuzzy appearance and the flexibility of the junctions.

**Ca\(^{2+}\)-independent Annexin 2-Membrane Interactions in the Cell—How relevant is the non-conventional (Ca\(^{2+}\)-independent and H\(^+\)-dependent) annexin 2 binding to membranes?**

Cytosolic pH variations (7.4–6.1) have been observed during cell cycle and differentiation of Dictyostelium (55), in swelling cardiomyocytes (56) and in epithelial cells (57). It is attractive to speculate on the role of two types of binding in the cells. A balance between the Ca\(^{2+}\) and H\(^+\) concentrations might be important for annexin 2 binding to membranes by these two distinct mechanisms. The Ca\(^{2+}\)-mediated binding mechanism could participate close to the Ca\(^{2+}\) channels near the exocytotic sites, and the H\(^+\)-mediated binding could be more important for other membranes (i.e. endosomes). Moreover, both mechanisms might induce particular bridging properties: more rigid with Ca\(^{2+}\) and more flexible with H\(^+\). Along with this line, the observed invagination of liposomes and the appearance of multilamellar structures at low pH resemble the acidic endocytic prelysosomal derived vesicles defined as multilamellar and multivesicular bodies (58, 59). It is also interesting to note that the arachidonic acid-induced fusion of chromaffin granules aggregated by annexins 7 and 2 is more efficient at pH 6 than at pH 7 (32, 60, 61). Recently, the presence of annexin 2 in PS-rich exosomes derived from the multivesicular bodies from dendritic cells has been described (62) as well as in phagosomes and phagolysosomes (63), particularly acidic organelles. These data raise the possibility that the H\(^+\)-mediated non-conventional annexin 2-membrane binding and aggregation could be involved in some steps of the endocytic pathway.

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