Ca\(^{2+}\)-dependent membrane interaction has long been recognized as a general property of the annexin (ANX) family of proteins. More recently, it has become clear that ANXs can also undergo Ca\(^{2+}\)-independent membrane interactions at mildly acidic pH. Here we use site-directed spin labeling in combination with circular dichroism and biochemical labeling methods to compare the structure and membrane topography of these two different membrane-bound forms of ANX12. Our results reveal strong similarities between the solution structure and the structure of the Ca\(^{2+}\)-dependent membrane-bound form at neutral pH. In contrast, all Ca\(^{2+}\)-independent membrane interactions tested resulted in large scale conformational changes and membrane insertion. Pairs of spin labels that were in close proximity across the interface of different domains of the protein in both the soluble and Ca\(^{2+}\)-dependent membrane form were >25 Å apart in the Ca\(^{2+}\)-independent membrane-bound form. Despite these major conformational changes, the overall secondary structure content did not appear to be strongly altered and ANX12 remained largely helical. Thus, Ca\(^{2+}\)-independent membrane interaction leads to massive refolding but not unfolding. Refolding did not occur at low pH in the absence of membranes but occurred within a few seconds after phospholipid vesicles were added. The phospholipid composition of the vesicles was an important modulator of Ca\(^{2+}\)-independent membrane interaction. For example, cardiolipin-containing vesicles induced Ca\(^{2+}\)-independent membrane interaction even at near neutral pH, thereby raising the possibility that lipid composition could induce relatively rapid Ca\(^{2+}\)-independent membrane interaction in vitro.

The annexins (ANXs) are a family of Ca\(^{2+}\) and membrane-binding proteins that often comprise up to 1% of the total intracellular protein. Their high degree of conservation, the lethality of some ANX knockout mice (2), and the emerging class of ANX-related diseases (annexinopathies) indicate that ANXs play important roles in vivo (3). Many membrane-related functions have been proposed for the different ANXs, including roles in membrane trafficking and fusion, ion channel formation, and cell adhesion (1). The likely importance of membrane interaction for ANX function has spurred general interest in attempting to understand the various modes and mechanisms by which ANXs interact with membranes. Recently it has become clear that ANXs can have at least two separate modes of membrane interactions: one is Ca\(^{2+}\)-dependent whereas the other one is Ca\(^{2+}\)-independent.

Of these two forms, the Ca\(^{2+}\)-dependent membrane interaction has been investigated most extensively and it has been shown that Ca\(^{2+}\) induces peripheral membrane binding of ANXs. According to electron microscopy (EM) (4), atomic force microscopy (5, 6) and site-directed spin labeling (SDSL) (7) analyses, the picture emerges that the structure of this membrane-bound form is closely related, but presumably not completely identical, to that of soluble ANXs. For example, EM and atomic force microscopy studies revealed a clear domain organization with overall shapes and dimensions that are similar to those observed in the x-ray structures of the soluble forms. As illustrated with the x-ray structure of “soluble” ANX12 in Fig. 1, ANXs typically contain four highly homologous repeats, each of which is made up of a 4-helical bundle (helices A, B, D, and E) and a fifth helix (helix C) that runs perpendicular to the bundle (Fig. 1).

The arrangement of these domains results in a somewhat curved molecule that contains a concave and a convex side (see Fig. 1). It is believed that Ca\(^{2+}\)-dependent membrane binding is mediated by loop regions between helices A and B, and between D and E located on the convex side of ANXs. However, because of a lack of high-resolution structural information in the presence of phospholipid bilayers, it is not clearly understood to what extent this membrane interaction induces subtle conformational changes.

Although ANXs were originally believed to be strictly Ca\(^{2+}\)-dependent membrane-binding proteins, in recent years there has been mounting evidence of additional ANX-membrane interactions that do not appear to be mediated by Ca\(^{2+}\). Numerous reports have demonstrated that certain cell types have a substantial pool of ANXs that cannot be removed from membranes with Ca\(^{2+}\)-chelating agents. Presently, it is not fully understood what factors confer this Ca\(^{2+}\)-independent membrane localization of ANXs. However, Ca\(^{2+}\)-independent membrane interactions of ANXs can be induced in vitro by triggers such as lowering the pH (8–11). Structural information for this type of membrane interaction came initially from SDSL anal-
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Fig. 1. Crystal structure of ANX12. Monomer (22) as seen from the side view (top) and concave view (bottom). The amino-terminal domain is gray, and each of the four repeats in the core domain is represented by a different color. Each repeat contains five helices (A–E). Ca²⁺-dependent binding is mediated by sites on the bottom of the molecule in the upper panel and into the PAGE in the lower panel. The locations of the positions that were mutated to cysteines and spin-labeled are highlighted by space-filling models of the amino acid side chains.

Fig. 2. Structure of side chains of R1 and R1'.

Analysis of a helical hairpin region of soluble ANX12 (D-E helical hairpin in repeat 2, Fig. 1). Upon pH-dependent membrane interaction, it was shown that this region undergoes a major inside-out refolding, resulting in the formation of a transmembrane helix that lines an aqueous cavity (11). These data could have important physiological implications as they demonstrate that soluble ANX12 can be reversibly converted into a transmembrane protein that appears to have the structural architecture of an ion channel. The notion of an ion channel-like structural reorganization of membrane-inserted ANX12 has since been confirmed through biochemical and electrophysiological analyses (10). However, the full extent to which conformational changes is not yet clear, because the original SDSL nitroxide scan characterized the structure of less than 10% of the protein.

To address this question and to compare the overall structure and fold of ANX12 in solution and its different membrane-bound states, we used a combination of methods. Membrane insertion was probed using biochemical methods. We used circular dichroism (CD) for global secondary structural analysis and SDSL (for review see Hubbell et al. (12)) to test for specific domain movements and reorganization. For the latter, we introduced pairs of cysteines at the interface of domains I and IV (residues 77 and 265) or at the interface of domains II and III (residues 113 and 241) (see Fig. 1). These cysteines were then labeled with a thiol-specific reagent to give the nitroxide-containing side chain R1 (Fig. 2). Because two spin labels located less than 25 Å from each other results in characteristic dipolar broadening of the EPR line shapes, the two doubly labeled derivatives of ANX12 (α-carbon distances are 8.3 and 8.8 Å, respectively) are sensitive indicators by which to evaluate overall structure and inter-domain packing interactions.

Using this combined approach, we present new evidence that pH (ranging from 7.4 to 4.5) in the absence of phospholipids, did not affect the overall secondary structure of ANX12, and that inter-domain interactions appeared to remain largely unchanged. Similarly, Ca²⁺-dependent membrane interaction did not appear to cause major alterations in the overall structure, although the inter-domain contacts appeared to become somewhat tighter and more rigid than they had been in solution. In contrast, interactions with membranes resulted in major domain reorganization at all pH values at which Ca²⁺-independent ANX-membrane binding occurred. Despite these large scale conformational changes detected by site-directed spin labeling, the overall amount of secondary structure remains similar based on CD measurements. Thus, Ca²⁺-independent membrane insertion must result in large scale protein refolding rather than nonspecific unfolding.

In an effort to understand how membrane insertion could be triggered in vivo, we investigated factors that modulate Ca²⁺-independent membrane interactions. We found that Ca²⁺-independent membrane interaction was strongly modulated by phospholipid composition and required major protein refolding. For example, ANX12 bound to cardiolipin (CL)-containing vesicles in a Ca²⁺-independent manner at near neutral pH. Together with the finding that ANX12 rapidly inserts into membranes, these data raise the interesting possibility that Ca²⁺-independent membrane interaction could occur in vivo, where it could be triggered either by transient changes in pH and/or changes in lipid composition.

EXPERIMENTAL PROCEDURES

Expression and Purification—Using the cysteine (−) clone (previously used in a number of studies (7, 11)) as a starting material, specific single mutations A77C and S241C were introduced in ANX12. The Clontech method was used to introduce additional mutations into A77C to create the double mutant A77C/K265C. The Cys-113 S241C mutant was created by removing the small NcoI/EcoRI fragment from S241C and replacing it with the small NcoI/EcoRI fragment of ANX12 pSE420-mmr33H, which contained the endogenous Cys-113 (13). The nucleotide sequences were verified by sequencing the entire gene. The ANX12 mutants were expressed in recombinant bacteria and purified by reversible Ca²⁺-dependent binding to phospholipid vesicles followed by column chromatography, according to previously published protocols (7, 14). ANX12 concentration was determined by absorbance (ε₅₉₀ = 12880 M⁻¹ cm⁻¹).
**Spin Labeling.**—Protein modification was accomplished by previously described methods (7, 14). Briefly, for R1 labeling of proteins, Site-1 thiol was removed from the buffer by size exclusion chromatography (PD10 column, Amersham Biosciences) and eluted with a buffer containing 20 mM Heps, 100 mM NaCl, and 1 mM EDTA (pH 7.4). The ANX12 mutants were reacted with a 5-fold excess of either R1 spin label 3-methanethiosulfonylmethyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrolyl-1-yl radical, or a mixture of R1 and R1' (diamagnetic analogue (15)) spin label (1:3 molar ratio) at room temperature (Fig. 2). The reactions were allowed to continue for 2 h and unreacted spin label was removed by size exclusion chromatography (PD10 column, Amersham Biosciences). For solution spectra, samples were first concentrated using YM-10 microconcentrator (Millipore).

The following preparations of lipids were obtained from Avanti Polar Lipids (Alabaster, AL): phosphatidylcholine/serine (PS, bovine brain, catalog number 800437), phosphatidylcholine/PC, egg yolk, catalog number 850355), and cardiolipin (bovine heart, catalog number 840012). Large unilammelar vesicles of the following composition (weight/weight) were extruded through a 100-nm membrane.

Unless otherwise indicated, the standard EPR experiments were performed using ~30 μg of spin-labeled ANX12 in 100 mM buffer at the appropriate pH (sodium acetate for pH 4.0–5.5, MES for pH 6.0–6.5, and Tris-Cl for pH 7.4) in either the presence or absence of phospholipid vesicles. For rapid kinetic measurements, membrane interaction was initiated at room temperature by mixing labeled ANX12 (30 μg) with an equal volume of vesicles (approximately 30 mM phospholipid), both equilibrated in 100 mM sodium acetate buffer at the indicated pH.

For Ca2+-independent membrane association the general co-pelleting protocol was followed. Briefly, 30 μg of protein was incubated with 1 ml of buffer (pH 4–7.4, as described above) plus 1 mM EDTA and 600 μg of phospholipid vesicles. Thus, the final concentration of ANX12 was ~1 μM resulting in a protein to lipid ratio of ~1:1000. The sample was incubated for 10 min at room temperature and vesicles along with bound protein were pelleted by centrifugation. In certain experiments at neutral pH, binding of ANX12 was induced by Ca2+ (1 mM). EPR experiments were recorded ~5 min after the sample was prepared.

**EPR Spectroscopy.**—X-band EPR spectra were obtained using a Bruker EMX spectrometer (fitted with a loop-gap resonator or the ER4119 HS cavity). Kinetics were obtained using a rapid mixing device connected to a Varian E-109 spectrometer fitted with a loop-gap resonator (7, 17). EPR spectra were taken at room temperature using standard settings (7, 11, 18). Scan width for EPR spectra was 150 gauss.

**Circular Dichroism.**—The far UV CD spectra of ANX12 was obtained as described under Experimental Procedures. The far UV CD spectra of ANX12 was recorded in solution at pH 7.4 (green trace) or 4.5 (black trace), and in the presence of either PS/PC (red trace) or CL/PC (blue trace) vesicles at pH 4.5 in the absence of Ca2+, as described under "Experimental Procedures.”

**RESULTS**

**Membrane and pH-dependent Changes in ANX12 Conformation.**—Previous studies showed that ANX12 exists in three structural forms: a soluble monomer, a Ca2+-dependent peripheral membrane-bound trimer at neutral pH, and a Ca2+-independent transmembrane form at mildly acidic pH (11). We have taken a combined CD and SDSLS approach to probe for structural differences between these three forms.

First, we asked whether pH alone, in the absence of membranes, could result in structural changes. As shown in Fig. 3, the CD spectra of ANX12 in the absence of lipid at pH 7.4 and 4.5 (green and black, respectively) are superimposable and exhibit two minima at 208 and 222 nm, as expected for a highly helical structure. Thus, the overall secondary structure content of ANX12 in aqueous solution does not seem to be affected by changes in pH alone. Next, we asked how this structural content was affected by the presence of PS- and CL-containing vesicles, with which ANX12 interacts at moderately acidic pH (11). We have taken a combined CD and SDSLS approach to probe for structural differences between these three forms.

To determine whether structural changes induced by membrane interaction are merely localized, or whether large scale refolding occurs that ultimately results in a very different structure with similar helical content, we employed an SDSLS approach as described in the Introduction. We prepared two different mutants of ANX12, each of which contained a pair of R1-labeled cysteine residues introduced across the interface between domains I/V (77R/I265R1) and II/III (113R/I241R1), as defined by the crystal structure of the soluble form of ANX12 (22). By monitoring distances between these pairs of spin labels, we were able to determine whether global conformational differences existed between the various states of ANX12. Fig. 4
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Fig. 4. The effects of phospholipid, pH, and Ca\(^{2+}\) on the EPR spectra of double-labeled mutants of ANX12. ANX12 containing double cysteine mutations at position 113/241 (left panel) or position 77/265 (right panel) were labeled with either R1 (black traces) or a mixture of R1 and an excess of non-paramagnetic R1' (red traces), as described under “Experimental Procedures.” The EPR spectra of these double-labeled mutants were then recorded at pH 7.4 (upper box) or pH 4.0 (lower box), either in solution (A, D, G, and J) or bound to phospholipid vesicles under the following conditions: Ca\(^{2+}\) plus PS/PC (B and H); Ca\(^{2+}\) plus CL/PC (C and I); PS/PC in the absence of Ca\(^{2+}\) (E and K); CL/PC in the absence of Ca\(^{2+}\) (F and L). The scan width was 150 gauss.

As expected from the x-ray structure (Fig. 1), the EPR spectra for both 113R1/241R1 and 77R1/265R1 at pH 7.4 in solution indicate close proximity of the spin-labeled sites (Fig. 4, A and G, black trace). Such close proximity is indicated by the broad line shape features and the overall breadth of the spectra that exceed 100 gauss for both derivatives. As outlined under “Experimental Procedures,” the effect of line broadening because of dipolar spin-spin interaction can conveniently be obtained by comparing EPR spectra from fully R1-labeled protein with protein labeled with a mixture of R1 and an excess of the non-paramagnetic reagent R1'. As shown in Fig. 4, the EPR spectra of the R1/R1' mixture (red traces) are strikingly different from those of the fully R1-labeled form. Most notable is the strongly increased signal intensity of the R1/R1'-labeled proteins, a feature that is the direct consequence of reduced line broadening in these cases (compare red and black traces in Fig. 4, A and G). The strong spin-spin interaction is in agreement with the proximity of the respective sites in the crystal structure (also see below). Interestingly, in the absence of membranes and with a change in pH from 7.4 to 4.0, this strong spin-spin interaction is still retained for 113R1/241R1 (compare Fig. 4, A and D) or 77R1/265R1 (compare Fig. 4, G and J) suggesting that the distance between the sites remains very similar. Thus, together with the CD data, the SDSL analysis demonstrates that, in solution, ANX12 does not undergo global unfolding or refolding at acidic pH (between pH 7.4 and 4.5).

Next, we asked whether the presence of PS and PC containing vesicles could induce pronounced structural changes. As can be seen in Fig. 4, E and K, the interaction of ANX12 with PS and PC containing vesicles at pH 4 in the absence of Ca\(^{2+}\) resulted in completely different EPR spectra when compared with those of the protein in solution. One of the most noticeably different features is the complete loss of spin-spin interaction, as evidenced by the similarity of the EPR spectra from the fully R1-labeled (Fig. 4, E and K, black traces) and the mixed R1/ R1'-labeled derivatives (Fig. 4, E and K, red traces). Thus, major conformational changes must occur that move the pairs of spin labels outside of the distance range detectable by conventional SDSL (i.e. 25 Å). The notion of a significant and global structural change is further supported by the strongly altered line shapes of the R1/R1'-labeled spectra of soluble and membrane-bound protein. To confirm that this change in the line shape is not because of simple local unfolding, but rather is a consequence of membrane-induced refolding, we recorded the spectrum of 113R1/241R1 in the presence of a high concentration of urea, thus giving an indication of the EPR line shape expected for a completely unfolded protein. In the presence of urea, 113R1/241R1 had a completely different line shape with very sharp (1.93 gauss centerline width) and narrowly spaced lines, as one would expect for an unfolded protein (data not shown).

Thus, our combined CD and SDSL approach strongly argues that the binding of ANX12 to PS and PC containing vesicles results in global conformational changes that extend through all four repeats of ANX12. Interestingly, these conformational changes are not a consequence of simple unfolding; rather, the refolded, membrane-bound form has secondary structural content comparable with that of the soluble form.

To contrast these conformational changes with possible changes occurring in response to Ca\(^{2+}\), we recorded the EPR spectra of ANX12 bound to PS-containing membranes at pH 7.4 (Fig. 4, B and H). As can be seen from the comparison of the R1-labeled and R1/R1'-labeled double mutants, strong spin-spin interactions were observed for this Ca\(^{2+}\)-dependent membrane interaction. Thus, as in solution, the two pairs of sites are in close proximity. To obtain more detailed distance information, we quantified the effect of spin-spin interaction as outlined under “Experimental Procedures.” According to these simulations, the 77R1/265R1 mutant exhibits a single population of distances around 9 to 10 Å in the soluble and Ca\(^{2+}\)-dependent membrane-bound forms. This side chain distance (nitroxide to nitroxide) is in good agreement with the separation of the α-carbons (8.8 Å) in the crystal structure. Also, in the case of 113R1/241R1, we observed a major distance population around 9 to 10 Å. In addition, the simulations revealed a longer...
distance distribution (around 13 to 14 Å). This population appears to be slightly more populated in the soluble form. Although the two populations could arise from two states of the protein, it should be noted that it is not uncommon to observe multiple distance population as a result of different spin label conformations (12, 23). Regardless of the precise details, the distance analyses for the soluble and Ca\(^{2+}\)-dependent membrane-bound forms are very similar, suggesting little conformational changes in these regions.

**ANX12 Membrane Interaction Is Modulated by Lipid Composition**—Because low pH, in the absence of phospholipids (Fig. 4, D and J), did not create the massive structural changes observed following Ca\(^{2+}\)-independent interaction with PS/PC membranes at low pH (Fig. 4, E and K), it appears that phospholipids must facilitate this conformational change. Therefore, we performed preliminary screens wherein we tested a variety of vesicles with different phospholipid compositions for their ability to induce Ca\(^{2+}\)-dependent membrane interaction of ANX12.

These screens identified two phospholipid compositions that varied substantially in their ability to induce binding (and conformational changes, see below) in a Ca\(^{2+}\)-independent manner. In co-sedimentation assays, little or no interaction could be observed with vesicles containing only PC, whereas CL-containing vesicles induce Ca\(^{2+}\)-independent membrane interaction and refolding of ANX12 at nearly neutral pH. Because of these interesting factors, we have systematically characterized the interactions of ANX12 with vesicles composed of CL and PC (CL/PC: 2:1 w/w ratio) by CD, SDSL, and biochemical labeling as well as co-sedimentation assays.

To study the pH-dependent membrane association of ANX12 with CL/PC vesicles in the absence of Ca\(^{2+}\), we performed co-sedimentation assays (top panel of Fig. 5). Nearly 100% of the added ANX12 bound to vesicles at low pH and some interaction occurred even at neutral pH. Half-maximal binding was observed at pH \(\sim 6.5\). To test the membrane topography of ANX12 on CL/PC vesicles, we measured its reactivity with \(^{125}\)I-TID, a photoactivatable hydrophobic reagent that selectively partitions into the hydrophobic core of bilayers (10).

Previous studies by our own group and others, showed that this reagent reliably labels proteins exposed to the hydrophobic core of bilayers but not to peripheral membrane-bound or soluble proteins (10). The lower panel of Fig. 5 shows that \(^{125}\)I-TID labeling was observed at pH 6.5 and lower in the absence of Ca\(^{2+}\), indicating that membrane insertion occurs under these conditions.

Next, we investigated whether ANX12 also undergoes peripheral binding to CL/PC vesicles at neutral pH in the presence of Ca\(^{2+}\) in a manner similar to the previously documented interaction with PS/PC vesicles (7). In a co-sedimentation assay (Fig. 5, top panel), quantitative binding occurred in the presence of Ca\(^{2+}\). As previously reported for PS-containing vesicles (21), binding to CL/PC was highly cooperative and half-maximal binding was observed at \(\sim 30\) \(\mu\)M Ca\(^{2+}\) (data not shown). The stoichiometry of Ca\(^{2+}\)-binding in the presence of CL/PC vesicles was 8.8 \(\pm\) 0.2 mol of Ca\(^{2+}\)/mol of ANX12, as determined by previous methods using \(^{45}\)Ca\(^{2+}\) (21). We repeated the \(^{125}\)I-TID labeling experiments outlined above in the presence of Ca\(^{2+}\) at pH 7.4. As shown in Fig. 5, the Ca\(^{2+}\)-dependent form was not labeled thereby indicating peripheral binding as expected from previous experiments with other phospholipids.

In summary, this biochemical analysis suggests that ANX12 interacts with cardiolipin-containing membranes in much the same way as it did with PS-containing membranes, but with one noteworthy difference: Ca\(^{2+}\)-independent binding and insertion into the membranes occurred at higher pH values that approached the physiological range.

To structurally characterize the interaction of ANX12 with CL/PC vesicles, we next employed CD and SDSL analyses. Previous studies showed that ANX12 formed a homotrimer following Ca\(^{2+}\)-dependent binding to PS-containing vesicles (7). A similar trimer has been observed for ANX4 and ANX5 (24). To determine whether this trimer was formed under similar conditions on CL/PC vesicles, trimer formation was monitored by a previously employed experimental strategy using ANX12 labeled at position 132R1 with R1 (7). Position 132 is near the 3-fold axis of the trimer, and R1 side chains at this location are in close proximity and strong spin-spin interactions were observed in the trimer (22). The EPR spectrum for 132R1 bound to CL/PC vesicles at neutral pH in the presence of Ca\(^{2+}\) was broad with low amplitude, indicating strong spin-spin interactions because of trimer formation (Fig. 6, dashed line).

The EPR spectrum of the same sample, after the addition of EGTA to chelate Ca\(^{2+}\), had narrow line shapes with high amplitude, as would be expected for the monomer in solution (Fig. 6, black trace). These sets of spectra are essentially identical to previous studies of the interaction of ANX12 132R1 with PS/PC vesicles (7), under these conditions and consistent
with the formation of a trimer on CL/PC vesicles. These data again emphasize the structural similarities of ANX12 when bound to either PS/PC or CL/PC vesicles in the presence of Ca\(^{2+}\).

The structure of ANX12 bound to CL-containing vesicles also was probed by EPR analyses of the 77R1/265R1 and 113R1/241R1 spin-labeled mutants. The EPR spectra for the double mutants, when bound to CL/PC vesicles in either the presence of Ca\(^{2+}\) at neutral pH (Fig. 4, C and I) or at pH 4.0 in the absence of Ca\(^{2+}\) (Fig. 4, F and L), were nearly superimposable with spectra recorded under the same conditions using PS/PC vesicles. These data imply that both CL and PS induce similar structural changes in ANX12; i.e. ANX12 bound to either PS- or CL-containing vesicles in the presence of Ca\(^{2+}\) appears to have a backbone fold similar to that observed in the crystal structure, and dramatically refolds following Ca\(^{2+}\)-independent binding at low pH. CD analysis also failed to detect any significant differences between ANX12 bound to either PS/PC or CL/PC vesicles at mildly acidic pH (Fig. 3).

To determine whether all of the pH-dependent membrane interactions that we observed for ANX12 require conformational changes, we systematically recorded the EPR spectra of the 113R1/241R1 derivative in the presence of different phospholipids at a variety of pH values in the absence of Ca\(^{2+}\). Representative EPR spectra obtained at different pH values in solution or in the presence of PS/PC or CL/PC are shown in Fig. 7, A–C.

The most striking feature in the EPR spectra of 113R1/241R1, caused by the structural reorganization induced by binding to membranes, is the increase in the signal amplitude resulting from loss of the spin-spin interaction. For convenience, we chose the signal amplitude of the central resonance line of the 113R1/241R1 derivative as a measure of the conformational changes induced by pH and phospholipid. The results obtained from this type of analysis are shown in Fig. 7D. In solution, no changes in signal amplitude were observed as a function of pH (Fig. 7, A and D, diamonds). Similarly, no changes were observed in the EPR spectra of the 113R1/241R1 derivative in the presence of PC vesicles at pH 4 (Fig. 7D, red stars), in agreement with our finding that ANX12 does not interact with these vesicles.

In the presence of PS/PC at pH 6.5 and higher, the signal amplitude was similar to those in solution, but increased as the pH was lowered with a half-maximal change at pH ~5.8 (Fig. 7, B and D, green circles). Although similar results were obtained in the presence of CL/PC; effects of the membranes were noted at significantly higher pH than with PS/PC. With CL/PC, the signal amplitude increased to nearly half-maximal value at approximately neutral pH (Fig. 7, C and D, blue circles). It appears, therefore, that ANX12 is able to interact with CL-containing membranes and to undergo major conformational changes even at physiological pH. It should be noted that the pH curves for binding of ANX12 to CL/PC vesicles (Fig. 5) are very similar to the curves for pH-dependent changes in signal amplitude observed for the 113R1/241R1 double mutant (Fig. 7). Thus, we observed conformational changes under all conditions in which we observed pH-dependent and Ca\(^{2+}\)-independent interactions with membranes containing cardiolipin. Similar conclusions apply to PS/PC membranes (Fig. 7 and Ref. 10).

Kinetics of Ca\(^{2+}\)-independent Membrane Interaction—To further characterize the mechanism of Ca\(^{2+}\)-independent membrane interaction, and to test whether ANX12 might be able to respond rapidly to possible pH or other triggers in vivo, we determined the kinetics of interaction. The kinetics of the conformational change that occur when ANX12 interacts with membranes in a Ca\(^{2+}\)-independent manner at low pH were monitored using the signal amplitude of the central resonance line of the 113R1/241R1 mutant as reporter. The amplitude as a function of time, after rapid mixing of the 113R1/241R1 mutant with PS/PC vesicles at low pH, is shown as a representative example in Fig. 8.

As shown in Fig. 8, membrane interaction occurs on the second time scale with a half-life of ~3 s. It should be emphasized that the method used to analyze these kinetic changes measures conformational changes and does not unambiguously establish the topography of the protein on the membrane. While additional experiments are needed to resolve this point, the observed membrane-induced changes in ANX12 were rapid relative to other well studied model proteins, e.g. colicin E1 adsorbed to and inserted into membranes with half-times of 6 and 127 s, respectively (25).
the x-ray structure of soluble ANX12 reveals several interactions that may be used to postulate some of the basic requirements for the membrane insertion that we have detected. In-
It is conceivable that at least some of such triggers would also give rise to conformationally altered, membrane-inserted protein.

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J. Mario Isas, Darshana R. Patel, Christine Jao, Sajith Jayasinghe, Jean-Philippe Cartailler, Harry T. Haigler and Ralf Langen

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