Annexins are soluble proteins that bind to membranes in the presence of Ca\(^{2+}\). Crystal structures have been determined for some soluble forms, but little is known about the important membrane-bound state. We employed site-directed spin labeling to demonstrate that 1) annexin XII assumes a trimer configuration similar to the crystal structure when bound to bilayers under physiological conditions; 2) trimer assembly on bilayers is remarkably rapid, occurring on a millisecond time scale, whereas subunit exchange requires hours; and 3) different annexins can mix to form heterotrimers. The rapid assembly and heterotrimer formation have important implications concerning the cellular functions of annexins.

Annexins are a ubiquitous family of structurally related proteins that share the common property of reversible Ca\(^{2+}\)-dependent binding to membranes containing phosphatidylsersine. There are at least 10 annexin genes in mammals, and each cell type expresses several different gene products. Many biological roles have been proposed for these proteins, including vesicle trafficking, membrane fusion, and ion channel formation. Although annexins are monomers in solution in the absence of Ca\(^{2+}\), they exist in a variety of quaternary states in crystals, including monomers (2), dimers (3), trimers (4, 5), and hexamers (6). Despite the wealth of information on crystal structures, including monomers (2), dimers (3), trimers (4, 5), and hexamers (6), the annexin structure is a bilayer-spanning hexamer (6) similar in topography.

High resolution three-dimensional crystal structures for the soluble forms of several annexins have been determined (1). Each structure shows the characteristic annexin fold, with binding sites that appear to coordinate Ca\(^{2+}\) jointly with phospholipid head groups and mediate peripheral membrane association (1). Although annexins are monomers in solution in the absence of Ca\(^{2+}\), they exist in a variety of quaternary states in crystals, including monomers (2), dimers (3), trimers (4, 5), and hexamers (6). Despite the wealth of information on crystal structures of the soluble forms, little is known about the membrane-bound state of annexins, information that is critical to evaluating proposed biological functions. Models for membrane-bound annexins include monomers, trimers, and hexamers with protein structures similar to those found in various crystals (Fig. 1). Data bearing on the membrane-bound structure consists primarily of electron microscopy images of stained samples of annexins on supported monolayers. These images have been interpreted as extended lattices of trimers (7, 8), but the data are of low resolution and were obtained under negative stain conditions on monolayer rather than bilayer surfaces. The distinction between a monolayer and bilayer is important especially because one current model of membrane-bound annexin structure is a bilayer-spanning hexamer (6) similar in structure to the annexin XII hexamer in the three-dimensional crystals (see Fig. 1). Chemical cross-linking studies of annexins V (9) and XII (10) bound to bilayers detected both trimers and hexamers, but limitations of the method make it difficult to draw definitive conclusions regarding multimerization or topography.

To obtain high resolution information on the structure of annexin XII bound to phospholipid bilayers under physiological conditions, we introduce a site-directed spin labeling approach that permits the study of the static structure on the membrane as well as the kinetics of its assembly. The general strategy of site-directed spin labeling involves the introduction of a unique cysteine residue that can be modified with a sulphydryl-selective nitroxide reagent. The present studies use a methanethiosulfonate reagent that generates the spin-labeled side chain designated R1 (11, 12) (see Structure I). The electron paramagnetic resonance (EPR) spectrum of R1 in a folded protein is sensitive to the mobility of the nitroxide. A comprehensive analysis of T4 lysozyme showed that it is possible to resolve loop, helix surface, tertiary contact, and buried sites in the folded protein on the basis of R1 mobility (13). In addition, the interspin distance between two R1 side chains can be estimated for distances up to about 25Å (14–17). In an isotropic sample containing mobile nitroxides, spin-spin interaction gives rise to a broadening of the spectral features, with a concomitant decrease in intensity (17). In the present work, we place R1 side chains at specific sites in annexin XII and employ both mobility and spin-spin interactions to analyze the structure of the membrane-bound form.

EXPERIMENTAL PROCEDURES

Specific mutations were introduced into the annexin XII pSE420-mrp33H plasmid (10) using the single-site mutagenesis kit from CLONTECH (Palo Alto, CA). First, two native cysteines in annexin XII were replaced with alanines (C113A/C302A). The C113A/C302A mrp33H.09 plasmid was used as the starting point for all the mutations reported herein. The following single mutations were introduced into C133A/C302A: R16C, K27C, I29C, K132C, E163C, D188C, S228C, and L260C. The CLONTECH method was used to introduce additional mutations into R16C and K27C to create two double mutants, R16C/E163C and K27C/D188C. One final double mutant, I29C/S228C, was created by removing the small Blp1HindIII fragment from I29C and replacing it with the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviation used is: EPR, electron paramagnetic resonance.
with the small Blp I/Hind fragment of S228C. All mutations were confirmed by DNA sequencing using a Sequenase 2.0 kit (Amersham Pharmacia Biotech).

The annexin XII mutants were expressed in recombinant bacteria and purified by reversible Ca\(^{2+}\)-dependent binding to phospholipid vesicles followed by column chromatography according to previously published protocols (10), except that dithiothreitol (2 mM) was added to all the buffers. The CD spectrum and Ca\(^{2+}\)-dependent phospholipid binding properties of cysteine-less annexin XII were indistinguishable from those of wild type annexin XII (data not shown).

Dithiothreitol was removed from the buffer in which the isolated proteins were stored by size exclusion chromatography (P-10 columns) immediately before modification of introduced cysteines with (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate under the following conditions: 2 h at room temperature, 10× molar excess reagent, 20 mM HEPES buffer, 100 mM NaCl, pH 7.4. Unreacted reagent was removed by size exclusion chromatography (P-10 columns). Spin-labeled mutants of annexin XII are designated by giving the sequence position of the cysteine substitution followed by the code for the nitroxide spin label, R1.

Vesicles with a lipid composition of phosphatidylserine (bovine brain)/phosphatidylcholine (egg yolk) in a 2/1 molar ratio were prepared according to the Reeves/Dowben protocol (18). The lipid:protein molar ratio in EPR experiments was typically 500:1.

EPR experiments were performed by adding spin-labeled annexin XII mutants (~30 μg of protein) to buffer (20 mM HEPES, 100 mM NaCl, pH 7.4)-containing vesicles. In certain experiments, complete binding of annexin XII to the vesicles was induced by Ca\(^{2+}\) (1 mM). EPR spectra were obtained using a Varian E-109 spectrometer fitted with a loop gap resonator (19). All spectra were obtained at 2 mW incident microwave power using a field modulation of ~1 G.

RESULTS AND DISCUSSION

To discriminate between the models shown in Fig. 1, R1 was introduced at sites in annexin XII indicated in Fig. 2. Site 132 was selected because R1 residues at this position would be in close proximity with strong spin-spin interaction in the trimer or hexamer but not in the monomer (Fig. 2A). As an additional test for the formation of trimers or hexamers, the double mutants 27R1/188R1 and 16R1/163R1 were prepared (see "Experimental Procedures"). Within a monomer, the residues in each pair are far apart (>20 Å), but in a trimer they lie in close proximity at a contact face. Finally, to distinguish trimers from hexamers, 260R1 and double mutant 29R1/228R1 were prepared (Fig. 2B). In the hexamer, residue 260 is within 5 Å of a symmetry-related residue 260 in the opposing trimer. Likewise, residues 29 and 228 in opposing trimers are within 5 Å of each other in the hexamer but are far apart in the monomer or trimer. Thus spin-labeled annexins 260R1 and 29R1/228R1 are expected to show spin-spin interaction only in the hexameric state.

In the presence of phospholipid vesicles but without Ca\(^{2+}\), annexin XII exists as a monomer in solution (10). Under these conditions, the EPR spectra for 132R1, 16R1/163R1, and 27R1/188R1 are similar, having narrow line shapes characteristic of R1 with a high mobility (Fig. 3A, blue traces). In each case, Ca\(^{2+}\) addition triggers annexin binding to the vesicles, with a dramatic decrease in EPR spectral amplitude (Fig. 3A, red traces). The decrease in amplitude is a consequence of spectral broadening, due to strong spin-spin interactions in the membrane-bound structure, as can be seen in the spectra at high gain (Fig. 3A, black, dashed traces). The existence of these specific interactions rules out the monomer model but is con-

![Fig. 1. Models for the membrane-bound annexin complex. The monomer and trimer are adsorbed on the bilayer surface, whereas the hexamer is inserted hydrophilically by inducing micelle-like structures in the bilayer (6).](image1)

![Fig. 2. Structure of the annexin XII hexamer in crystals. The structure is that of a dimer of trimers (6). A, top view showing one trimer with each monomer in a different color. Sites selected for introduction of R1 to detect trimer formation are indicated. B, side view of the hexamer, with each trimer in a different color. Sites selected for introduction of R1 to detect hexamer formation are indicated.](image2)
Membrane-mediated Assembly of Annexin Trimmers

Fig. 4. The amplitude of the central resonance line of membrane-bound 132R1 mixed with unlabeled, Cys-less annexin XII before the addition of Ca$^{2+}$, plotted as a function of the fraction of labeled protein, $f$. The green, red, and blue lines represent predictions for a hexamer, trimer, and dimer, respectively (see text). For a structure with $N$ subunits, the fraction $F_i$ that have $i$ spin-labeled subunits when a fraction $f$ of the subunits is labeled is, according to the following binomial theorem.

$$F_i(f, N) = \frac{N!}{(N-i)!i!} f_i(1-f)^{N-i}$$

(Eq. 1)

For spins in close proximity, such as for two or more 132R1 subunits in a trimer or hexamer, the spin interaction is strong and the spectral amplitude is small compared with that of noninteracting spins. To a good approximation, the signal amplitude of trimers or hexamers containing two or more spin-labeled subunits may be taken as zero. Thus, the amplitude of the central resonance line in a population of oligomers is just

$$A(N, f) = c F_1(1, f, N) = c N! \left[ \frac{1}{N-1} \right] f (1-f)^{N-1}$$

(Eq. 2)

where $c$ is a scaling constant, and $b$ is a constant that accounts for the inevitable presence of a small amount of signal amplitude at the position of the central resonance line even when spins are in strong interaction. This signal may arise, for example, from the presence of a small amount of free, unattached spin label or monomeric annexin. For the data shown in Fig. 4, $b = 1$, the value of $I$ when $f = 1$. In the present experiments, this amounts to only about 3% of the theoretical amplitude that would be obtained for the same concentration of noninteracting spins. Thus, the upper limit for the presence of monomer is 3%.

If the spectral broadening upon membrane binding is due to specific spin-spin interactions between monomers in an oligomer, the EPR spectra should be altered by mixing labeled with unlabeled monomer before Ca$^{2+}$ addition. For example, Fig. 4 shows a plot of the amplitude of the central resonance line for 132R1 versus $f$, the fraction of labeled monomer in the sample. Assuming random mixing of labeled and unlabeled monomers and strong interaction of spin pairs in the oligomer, the form of this plot may be predicted for various oligomer stoichiometries from the binomial theorem (see the legend of Fig. 4). The solid curves in the figure give the predicted variation of amplitude with $f$ for dimers (blue trace), trimers (red trace), and hexamers (green trace). As can be seen, the data are well fit by the trimer prediction. Taken together, the data of Figs. 3 and 4 provide strong evidence that the membrane-bound state triggered by Ca$^{2+}$ closely resembles that of the crystallographic trimer.

At high dilution with unlabeled, Cys-less annexin XII ($f = 0.1$), the EPR spectrum of 132R1 is that of a single R1 side chain in a trimer in the absence of spin-spin interaction (Fig. 3A, green trace). The line shape reflects a relatively high mobility for R1, consistent with the location of 132 in a loop facing a central cavity in the trimer structure (Fig. 2A). For the double mutants 16R1/163R1 and 27R1/188R1, the spectral line shapes in the trimer in the absence of spin-spin interactions were obtained as the algebraic sums of the spectra of the correspond-

Fig. 3. EPR spectra of R1 at the indicated sites in annexin XII, in the presence of phospholipid vesicles. All spectra for a given labeled protein are scaled to have the same second integral (same number of spins). In particular cases, the scaled spectra are multiplied by the indicated gain factor. Scan width for all is 150 Gauss. A, spectra of R1 at sites selected to detect trimer and hexamer formation. For each case are shown the spectrum without Ca$^{2+}$ (blue trace), the spectrum after the addition of 1 mM Ca$^{2+}$ (red trace), a high gain display of the spectrum after Ca$^{2+}$ addition (dashed, black trace), the spectrum in the membrane-bound state with Ca$^{2+}$ but with reduced or absence spin-spin interactions (green) (see text). The indicated gain factors are relative to the blue trace. B, spectra of R1 at sites selected to detect hexamer formation. The color code is the same as in A. C, spectra of annexin XII 132R1, with an excess of unlabeled human annexin V added before (green trace) or 10 min after (red trace) Ca$^{2+}$ addition. The molar ratio of annexin XII/annexin V was 1:9.

Consistent with a trimer (or hexamer) similar to that in Fig. 2 in the membrane-bound state.

The addition of Ca$^{2+}$ in the absence of membranes has no effect on the line shapes in solution (data not shown), demonstrating that the spectral changes triggered by Ca$^{2+}$ are in fact related to assembly of a structure on the bilayer surface. Previous studies reported the formation of oligomers in solutions of Ca$^{2+}$ without membranes (10). However, much higher concentrations of Ca$^{2+}$ and protein were employed in those studies.

If the spectral broadening upon membrane binding is due to specific spin-spin interactions between monomers in an oligomer, the EPR spectra should be altered by mixing labeled with unlabeled monomer before Ca$^{2+}$ addition. For example, Fig. 4 shows a plot of the amplitude of the central resonance line for 132R1 versus $f$, the fraction of labeled monomer in the sample. Assuming random mixing of labeled and unlabeled monomers and strong interaction of spin pairs in the oligomer, the form of this plot may be predicted for various oligomer stoichiometries from the binomial theorem (see the legend of Fig. 4). The solid curves in the figure give the predicted variation of amplitude with $f$ for dimers (blue trace), trimers (red trace), and hexamers (green trace). As can be seen, the data are well fit by the trimer prediction. Taken together, the data of Figs. 3 and 4 provide strong evidence that the membrane-bound state triggered by Ca$^{2+}$ closely resembles that of the crystallographic trimer.

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(Eq. 1)

For spins in close proximity, such as for two or more 132R1 subunits in a trimer or hexamer, the spin interaction is strong and the spectral amplitude is small compared with that of noninteracting spins. To a good approximation, the signal amplitude of trimers or hexamers containing two or more spin-labeled subunits may be taken as zero. Thus, the amplitude of the central resonance line in a population of oligomers is just

$$A(N, f) = c F_1(1, f, N) = c N! \left[ \frac{1}{N-1} \right] f (1-f)^{N-1}$$

(Eq. 2)
Membrane-mediated Assembly of Annexin Trimers

The strong spin-spin interaction developed upon trimer formation in 132R1 affords an opportunity to monitor both the time course of trimer formation and subunit exchange between trimers. For example, Fig. 5A shows a trace of the central resonance amplitude after the stopped-flow mixing of 132R1 and vesicles in the presence of Ca$^{2+}$. The decrease in amplitude traces the development of spin-spin interaction, a process essentially complete within ~150 ms under the conditions employed. In fact, the amplitude decreases to approximately 50% of its final value within the ~4 ms dead time of the instrument (19). The speed of this process is remarkable considering that multiple protein-Ca$^{2+}$ lipid interactions must be formed in addition to protein-protein contacts between the three monomers. Although more extensive studies will be required to draw conclusions regarding the mechanism of assembly, these data provide the first illustration of the remarkable overall rapidity of the event, a point of potential significance to the biological function of annexins.

Fig. 5B illustrates the time course of subunit exchange between annexin XII trimers. In this experiment, vesicles containing pre-assembled 132R1 trimers in the presence of Ca$^{2+}$ were mixed with unlabeled Cys-less annexin XII. Initially, the EPR spectral amplitude is low due to spin-spin interaction within the trimers. Upon the addition of unlabeled protein, the amplitude increased over a period of about 2 h, reflecting a decrease in spin-spin interaction resulting from mixing of labeled and unlabeled protein. The final amplitude is that expected from the fraction of labeled monomer present, and the EPR line shape after complete mixing is essentially identical to that of 132R1 in a dilute trimer (Fig. 3B, green trace for 132R1). These data clearly establish that subunit exchange occurs on a time scale orders of magnitude slower than that of trimer formation.

In an analogous fashion, mixing of annexin XII with other members of the annexin gene family can be assayed. For example, Fig. 3C (green trace) shows the spectrum obtained by addition of Ca$^{2+}$ to a mixture of hydra annexin XII 132R1 and an excess of unlabeled human annexin V. This spectrum is indistinguishable from that obtained by addition of Ca$^{2+}$ to a mixture of annexin XII 132R1 and an excess of unlabeled annexin XII (Fig. 3A, green trace for 132R1). However, if annexin V is added 10 min after annexin XII 132R1 membrane binding, the strongly interacting spectrum of the annexin XII 132R1 homotrimer (Fig. 3C, red line) is obtained, due to the slow subunit exchange. The data clearly show that different members of the annexin gene family can form heterotrimers, an event not previously reported.

In summary, the data presented in this report directly demonstrate that 1) under the conditions tested, a trimer similar to that in the crystal structure forms the dominant population of annexin XII bound to lipid bilayers; 2) Ca$^{2+}$-triggered annexin XII trimer formation on the membrane surface can occur on the scale of milliseconds, raising the possibility of annexin involvement in rapid vesicle fusion events, such as synaptic transmission; and 3) despite their evolutionary distance, human annexin V and hydra annexin XII have retained the ability to cross-oligomerize. Since all mammalian cells express several different annexin gene products, we propose that the formation of heterotrimers be added to the list of possible regulatory elements in the annexin system.

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