Structure and Dynamics of a Helical Hairpin that Mediates Calcium-dependent Membrane Binding of Annexin B12*

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A wealth of high-resolution structural data has accumulated for soluble annexins, but only limited information is available for the biologically important membrane-bound proteins. To investigate the structural and dynamic changes that occur upon membrane binding, we analyzed the electron paramagnetic resonance (EPR) mobility and accessibility parameters of a continuous 30-residue nitroxide scan encompassing helices D and E in repeat 2 of annexin B12 (residues 134–163) while the protein was bound to phospholipid vesicles in the presence of Ca2+. A comparison of these data to those from a previously published study of the protein in solution (Isas, J. M., Langen, R., Haigler, H. T., and Hubbell, W. L. (2002) Biochemistry 41, 1464–1470) showed that the overall backbone fold for the scanned region did not change upon membrane binding. However, side-chains in the loop between the D and E helices were highly dynamic in solution but became essentially frozen in the EPR time scale upon binding to membranes. Accessibility measurements clearly established that side-chains in this loop were exposed to the hydrophobic core of the bilayer and provide the first evidence that a D–E loop directly participates in the Ca2+-dependent binding of annexins to membranes. Other localized changes showed that the D-helix became much less dynamic after membrane binding and identified quaternary contact sites in the membrane-bound homo-trimer. Finally, immobilization of the D–E loop upon contact with phospholipid suggests that the bilayer, which is normally very mobile on the EPR time scale, is immobilized in the head-group region by the annexin B12. This suggests that annexin B12 alters membrane structure in a manner that may be biologically significant.

Ca2+ plays an important regulatory role in many aspects of cellular life. Some of the most abundant Ca2+-sensing proteins in multicellular organisms belong to the annexin family of proteins. A hallmark of annexins is their ability to bind to phosphatidylserine-containing vesicles in a Ca2+-dependent manner. Many diverse functions have been proposed for the different members of the annexin family (1), and all rely on the ability of annexins to interact with membranes. Despite its obvious biological importance, relatively little detailed molecular information is available regarding the structure and dynamics of the membrane-bound form of the annexins. Images of two-dimensional crystals of annexins 4 and 5 on phospholipid monolayers obtained by electron crystallography indicated that the membrane-bound proteins had the same general shape as observed in x-ray crystal studies of the soluble proteins but lacked the resolution to draw further inferences (2, 3). Atomic force microscopy studies of annexin A5 on the surface of planar lipid bilayers reached similar conclusions (4).

In contrast to the limited information available for membrane-bound annexins, much has been learned about the structure of different annexins in their water-soluble form. Crystal structures have been solved for several annexins (5, 6), and the general picture emerges that all annexins might share the same overall fold. As illustrated by annexin B12 (7) in Fig. 1, annexins typically are made up of four highly homologous and conserved repeats. Each of these repeats contains a four-helical bundle and a fifth helix that runs perpendicular to the bundle (Fig. 1). Another common feature of annexins is a somewhat curved shape that gives rise to a convex and a concave side. The convex side of annexins contains eight interhelical loops that bind multiple Ca2+ ions when crystals are grown in the presence of high concentrations of Ca2+. Based on a crystal structure of annexin A5 in which one of these Ca2+ ions is coordinated by both the protein and by the head group of a soluble phospholipid analogue, annexins are proposed to bind to membranes via a Ca2+-bridge mechanism (8). This proposal is supported by studies of annexin A5 that used the fluorescence of a naturally occurring Trp to show that one of the eight loops, the A–B loop in repeat 3, is in direct contact with the membrane (9, 10). However, the involvement of the other binding sites has not been shown directly.

The current study employs site-directed spin labeling (SDSL) to determine structural features and molecular mechanism that allow annexin B12 to bind to bind to membranes in a Ca2+-dependent manner. This is an attractive model system that should provide insights into the general biophysical principles that govern the interaction between proteins and phospholipids. SDSL is based on the introduction of specific nitroxide groups into the protein sequence (11). This is typically done by modifying unique cysteine residues in the protein sequence to give the new side chain R1 (Structure 1).

The shape of the electron paramagnetic resonance (EPR) spectrum of the nitroxide-labeled protein reflects the mobility of the R1 side chain and can be used to provide information on local (12, 13) and backbone dynamics (14). Measurement of the...
EPR accessibility parameters to paramagnetic reagents in solution can be used to determine solvent accessibility of R1 in a soluble protein and to determine topography of a membrane protein (15). Secondary structure of a protein can be determined by nitroxide scanning experiments in which single native side chains are sequentially replaced with R1, and the EPR mobility and/or accessibility parameters are measured and analyzed for periodic changes as a function of sequence number.

Using SDS, we have recently studied the interaction of annexin B12 with membranes (13, 16–18). Annexin B12 is a dynamic protein that can reversibly adopt a number of conformations in response to changes in phospholipid, pH, and Ca\(^{2+}\). It is a monomer in solution, but in response to Ca\(^{2+}\), it rapidly forms a trimer on the surface of bilayers (16) that resembles the trimer in the crystallographic hexamer (7) composed of a dimer of trimers (Fig. 1, bottom). Annexin B12 also undergoes an “inside-out” global refolding (18) and forms a transmembrane pore by a Ca\(^{2+}\)-independent mechanism at mildly acidic pH (17, 19). The backbone fold of the transmembrane form is completely different from the fold of the Ca\(^{2+}\)-dependent trimer on the surface of membranes. It is not yet known whether transmembrane insertion is a general property of the annexin family of proteins but at least one other annexin, annexin A5, seems to insert into membranes in this manner (19).

In this report, we present a nitroxide scanning study in which every amino acid in the sequence from 134 to 163 of annexin B12 has been replaced by R1 one at a time (Fig. 1). We have previously used SDS to show that this region exhibits a structure in solution that is similar to the one observed in the crystal structure (15). However, at low pH and in the presence of phosphatidylserine-containing vesicles, this region undergoes a massive inside-out refolding (18) and is converted into a continuous transmembrane helix (17, 20). Herein, we show that the structure of the Ca\(^{2+}\)-dependent membrane-bound state is closely related to that of the solution structure, except with large changes in the dynamics of the D–E loop caused by direct contact with the phospholipid bilayer. The immobilization of this membrane-binding loop is likely to contribute to the stabilization of helix D, which also becomes significantly less dynamic upon membrane binding. Overall, little evidence was found for structural changes in helix E, except for an immobilization of sites on its outside surface. These changes are probably caused by quaternary contact resulting from trimer formation.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—A series of 30 single cysteine substitution mutants that correspond to residues 134–163 of annexin B12 were constructed by methods described previously (16, 17). All mutations were confirmed by DNA sequencing by using a Sequenase 2.0 kit (Amersham Biosciences). The annexin B12 mutants were expressed in recombinant bacteria and purified by reversible Ca\(^{2+}\)-dependent binding to phospholipid vesicles followed by column chromatography as described previously (16).

Spin-labeling and EPR Measurements—The methods used to modify the introduced cysteines with spin-label (1-oxy-2,2,5,5-tetramethylpyrroolidyl-3-methyl)methanethiosulfonate (a generous gift from Prof. K. Hideg, University of Pecs, Hungary) were as described previously (16). Spin-labeled mutants of annexin B12 are designated by giving the sequence position of the cysteine substitution followed by the code of the nitroxide spin-label, R1. Spin-labeled proteins were stored in dilute buffer at pH 7.4. Before use in EPR experiments, the spin-labeled proteins were concentrated in a Microcon YM-10 (Amicon) using a tabletop microcentrifuge at the recommended speed. Large vesicles with a lipid composition of phosphatidylserine (brain; Avanti Polar Lípides, Alabaster, AL) and phosphatidylcholine (egg yolk; Avanti Polar Lípides) at a 2:1 molar ratio were prepared according to Reeves/Dowben protocol (21).

EPR experiments were performed on spin-labeled annexin B12 mutants (30 µg protein) in buffer (20 mM HEPES and 100 mM NaCl, pH 7.4) plus phospholipid vesicles. The molar ratio of protein to lipid was 1:500, and the binding of annexin B12 to the vesicles was induced by Ca\(^{2+}\) (1 mM). EPR spectra were obtained by using a Varian model E-109 spectrometer fitted with a loop gap resonator (22). All spectra were...
obtained at 2 mW incident microwave power and a field modulation of ~1 G (1 G = 0.1 millitesla). For the accessibility measurements, the oxygen concentration was that of oxygen in equilibrium with air and the chelated nickel (NiEDDA) concentration was 3 mM.

RESULTS

EPR Spectra and R1 Mobility—To probe for local changes in structure and dynamics upon Ca$^{2+}$-dependent membrane binding, we generated 30 R1-labeled derivatives of annexin B12 (residues 134–163) and recorded the EPR spectra for both the soluble and membrane bound forms. Fig. 2, red traces, shows the EPR spectra for each R1-labeled annexin B12 derivative while bound to phospholipid vesicles composed of phosphatidylyserine/phosphatidylcholine (2:1) in the presence of Ca$^{2+}$ at neutral pH. The previously published (13) spectra of these derivatives in 30% sucrose solution are shown for comparison (Fig. 2, black trace). Under both conditions, the line shapes of the EPR spectra reflect only internal motions of the R1 side chain and local backbone fluctuations because the rotational motion of the protein was reduced by either binding to membranes or by the presence of sucrose. Thus, the two sets of spectra can be directly compared with reveal how Ca$^{2+}$-dependent membrane interaction affects local structure and dynamics.

As can be seen in Fig. 2, there are extended regions in which the EPR spectra for the solution and membrane-bound state are highly similar or nearly superimposable (Fig. 2, highlighted by black numerals). These regions include most of helix E, the loop region from residues 157 to 163, and parts of helix D, suggesting that, at least to some extent, the overall character of the protein fold was retained upon Ca$^{2+}$-dependent membrane binding. Nevertheless, significant spectral changes can be detected at certain positions (Fig. 2, highlighted by red numerals), especially in the interhelical loop (residue 142R1), which contains the putative membrane-binding region. These changes occurred only in the simultaneous presence of phospholipid vesicles and Ca$^{2+}$, because addition of either one alone caused no significant spectral changes (data not shown). The most dramatic effect of Ca$^{2+}$-induced binding to bilayers was observed at position 144R1. In solution, the nitroxide at this site was highly mobile, similar to R1 in flexible loops in other proteins (12). Membrane binding effectively freezes out this motion on the EPR time scale as indicated by the broadened lines, the increased separation between the outer peaks, and the concomitant decrease in signal amplitude (Fig. 2). This is graphically illustrated in Fig. 3, which shows a plot of the inverse of the central linewidth ($\Delta H_{\text{e}}^{-1}$) as a function of residue number. $\Delta H_{\text{e}}^{-1}$ is commonly used as a semiquantitative measure of mobility with a larger number generally indicating higher mobility (12). Fig. 3 shows that of all the positions scanned, 144R1 has the highest mobility in sucrose (black trace) but nearly the lowest mobility in the membrane-bound state (red trace). This effect is not limited to position 144R1 but extends to other sites around it. With the exception of 143R1, which is already immobilized in solution, all other sites in this loop region and the C-terminal region of helix D (residues 140R1 and 141R1) experience very strong immobilization upon membrane binding (Figs. 2 and 3). Thus, membrane interaction causes a pronounced large-scale suppression of mobility for D–E connecting loop region, whereas the loop

![Fig. 2. EPR spectra of annexin B12 containing the R1 nitroxide side chain at the indicated sites. All spectra were normalized to the same number of spins. The red traces present the EPR spectra of spin-labeled mutants bound to phospholipid vesicles in the presence of Ca$^{2+}$ as described under “Experimental Procedures.” Spectra recorded in solution (30% sucrose; black trace) were reported in a previous publication (13). For clarity of presentation, the amplitudes of some spectra were either increased or reduced by a scaling factor shown to the right of the spectra. The boxes denote positions in the D helix (positions 134–142) and the E helix (positions 146–156). Positions that are noted with red numbers had notable differences in the spectra of the protein in solution and the membrane-bound state (see text).](http://www.jbc.org/)

![Fig. 3. Sequence dependence of R1 mobility. $\Delta H_{\text{e}}^{-1}$ (inverse of the central linewidth) values of annexin B12 mutants in the Ca$^{2+}$-dependent membrane-bound form (red triangles) and the soluble form (black circles) are shown as a function of sequence position. The red shaded boxes on the left and right correspond to residues in the D and E helices, respectively, in the crystal structure of annexin B12 (7).](http://www.jbc.org/)
regions on the opposite (concave) side of the molecule (157R1–163R1) seem to be largely unaffected.

Although the overall immobilization was the most pronounced in the D–E interhelical loop regions, significant spectral changes were also observed in helix D (138R1–142R1), particularly toward the C-terminal region adjacent to the D–E interhelical loop region. To a much lesser extent, spectral changes also were observed at some sites in helix E (residues 153R1 and 156R1). To better understand the structural basis responsible for these EPR spectral changes, it is important to first consider the structure and dynamics of this region in solution. In solution, both helices exhibit periodic variations in mobility that are in agreement with their structure in the crystal. For example, helix surface sites (positions 137R1, 141R1, 149R1, and 153R1) correspond to local mobility maxima, whereas the buried residues (positions 135R1, 139R1, 147R1, 151R1, and 154R1) display minimal mobility. It is interesting that the overall mobility values in solution are significantly higher in helix D than in helix E (Fig. 3). Based on this differential mobility and other data, our previous study of this region in solution concluded that helix D was more dynamic and loosely packed than the longer helix E (13).

In the Ca$^{2+}$-dependent membrane-bound state, the mobility of residues 134R1 through 142R1 and 146R1 through 156R1 showed a periodic change in $\Delta H_{\text{O}_2}^{-1}$ that was again entirely consistent with a helical structure. For example, in the bound state residues expected to be buried on the inner face of helix D (135R1, 139R1) and helix E (147R1, 151R1, 154R1) were strongly immobilized and correspond to local minima for the $\Delta H_{\text{O}_2}^{-1}$ values (Figs. 2 and 3). Based on these mobility values, it therefore seems that no major conformational changes occurred. In the absence of conformational reorganization, the differences in mobility observed especially in helix D are therefore likely to reflect alterations in the overall dynamics. Indeed, the $\Delta H_{\text{O}_2}^{-1}$ values in Fig. 3 indicates a general suppression of R1 mobility at all sites in helix D, especially those close to the D–E interhelical loop region. Thus, helix D must have become significantly more rigid after Ca$^{2+}$-dependent membrane binding. We interpret this decreased mobility after membrane binding at all positions in helix D as being caused by tighter helix packing and reduced backbone motion (Fig. 2 and 3). It seems likely that this more rigid structure is induced by the strong ordering experienced by the D–E interhelical loop region, which is likely to serve as an anchor for the otherwise more dynamic helix D.

The EPR spectra and $\Delta H_{\text{O}_2}^{-1}$ values for most positions in helix E were very similar in solution and after membrane binding. Nevertheless, we noted two sites (153R1, 156R1) at which the R1 spectra reflect a decreased mobility membrane interaction (Fig. 2). It is interesting that both of these sites fall onto the outside surface of helix E and are located at a site of contact in the crystallographic trimer (Fig. 1). Thus, the most likely explanation for this immobilization is the formation of quaternary contacts between different subunits in a membrane bound-trimer.

Accessibility to Oxygen and NiEDDA and Structural Model of Membrane-bound Form—Data presented in the previous section indicated that the loop between the D and E helices became dramatically less dynamic after Ca$^{2+}$-dependent membrane binding (Fig. 2 and 3). Because this region is located on the convex surface of annexin B12, it is likely that it might directly interact with the membrane. To test this notion and to obtain more detailed structural information, we measured the accessibility parameters $\Pi(O_2)$ and $\Pi($NiEDDA) for R1 at each position in the scanned region of annexin B12. For a protein in aqueous solution, accessibility to these colliders depends on the local structure, in particular the degree to which R1 becomes buried in the protein. As a consequence, the periodic oscillations of both $\Pi(O_2)$ and $\Pi($NiEDDA) along a helix are largely in phase. For a membrane protein, topography is the most important factor in determining accessibility, because a site exposed to the membrane will exhibit strong accessibility to $O_2$, which partitions into the hydrophobic core of the membrane, but weak accessibility to the more hydrophilic NiEDDA (15).

Fig. 4, top, shows that $\Pi(O_2)$ and $\Pi($NiEDDA) were in phase in both helical regions of the membrane-bound protein, demonstrating that the helices behave like regions in a soluble protein and are therefore not in immediate contact with the phospholipid bilayer. Furthermore, the accessibility to $O_2$ and NiEDDA were high throughout the loop region from residue 157R1 to 163R1 as would be expected for a loop region that was not in membrane proximity. On the other hand, 142R1, 144R1, and 145R1 had high $\Pi(O_2)$, characteristic of an R1 residue that interacts with the bilayer. The contrast parameter $\Phi = \ln(\Pi(O_2)/\Pi($NiEDDA$))$ is useful for identifying nitroxide side chains that are exposed to bilayers with high $\Phi$ values corresponding to membrane exposure (15). Fig. 4, bottom, presents a plot of $\Phi$ as a function of sequence position in the scanned region of annexin B12 with the exception of positions 147R1, 151R1, 154R1, and 155R1, which were excluded from this analysis because the $\Pi$ values at these positions were near zero. Fig. 4, bottom, clearly identifies 142R1, 144R1, and 145R1 as sites that are directly exposed to bilayers (Fig. 4, bottom). In striking contrast to the high $\Phi$ values obtained for these three residues in the membrane-bound protein, low $\Phi$ values were measured (13) in solution for 142R1, 144R1 and 145R1 ($\phi = -0.87, -1.10$, and $-1.11$, respectively). The other residue in the loop between the two helices, 143R1, had moderate accessibility to both $O_2$ and
NiEDDA (Fig. 4, top) and a low \( \Phi \) value (Fig. 4, bottom) in the membrane-bound state, thereby indicating that it was not in direct contact with the bilayers. Thus, despite the fact that 143R1 is flanked by residues that interact directly with the bilayer, its side chain seems to face away from the bilayer and most probably is folded back into the protein. These data are consistent with the crystal structure of annexin B12, which indicates that the side chain of Thr-143 projects inward to a location between the D and E helices (see "Discussion").

**DISCUSSION**

Previous studies provided a wealth of high-resolution data on soluble annexins but only limited and lower-resolution data on the Ca\(^{2+}\)-dependent membrane-bound form of the proteins (5). The primary goal of this study was to determine the structural and dynamic changes that occur upon Ca\(^{2+}\)-dependent binding of annexin B12 to the membrane surface. Toward this end, the mobility and accessibility parameters of a nitroxide scan of positions 134 through 163 of annexin B12 were measured while the protein was bound to phosphatidylserine-containing vesicles in the presence of Ca\(^{2+}\), and these data were compared with previously published values for these positions determined while the protein was in solution (13). These data indicate that the overall backbone fold for the scanned region in the Ca\(^{2+}\)-dependent membrane-bound state is similar to the fold in solution as determined by EPR analysis but is totally inconsistent with the structure of the scanned region when the protein forms a transmembrane pore at low pH in the absence of Ca\(^{2+}\) (17, 18). However, it should be noted that the scanned region was not positioned in a region suitable to analyze the "hinge" motion proposed to occur when soluble annexins bind to bilayers in the presence of Ca\(^{2+}\) (23).

Although the overall fold was similar after membrane binding, some structural changes were noted in: 1) the loop between the D and E helices, 2) the D helix, and 3) sites of quaternary membrane-bound trimer. These changes and their implications with regard to the mechanism of Ca\(^{2+}\)-dependent membrane binding of annexins are discussed below.

This discussion is facilitated by referring to Fig. 5, which presents a structural model of the scanned region of annexin B12 based on x-ray crystallography. In constructing this figure, we also consider a published crystal structure of annexin A5 in complex with Ca\(^{2+}\), and glycerophosphoserine, an analogue of the polar head-group of phosphatidylserine (8). Based on this annexin A5 structure, we modeled Ca\(^{2+}\) (yellow sphere) and PO\(_4\) (green and red) into annexin B12 in Fig. 5, right, which represents the membrane-bound form of the protein. The Ca\(^{2+}\) and PO\(_4\) provide orientation with regard to the location of the bilayer, which would be expected to be horizontal and below the protein in Fig. 5. In addition to presenting the static x-ray structure, the backbone in Fig. 5 is color-coded to represent the dynamic properties of the protein based on \( \Delta H_{m}^{-1} \) values presented in Fig. 3.

By far the most striking effect of membrane interaction was the strong reduction of the dynamics in the D–E interhelical loop region. In solution (Fig. 5, left) this loop is very dynamic, but upon membrane interaction, it becomes highly structured and immobilized (Fig. 5, right). Based on differences in \( \Omega_{2} \) and NiEDDA accessibility (Fig. 4, bottom), it is clear that 142R1, 144R1, and 145R1 insert into a hydrophobic region of the bilayer, whereas 143R1 (also located in the loop) projects toward the core of the protein as expected from the crystal structure (Fig. 5). Thus, this study establishes a direct interaction between the membrane and the D–E loop and provides the first direct data that the D–E loop of any annexin participates in Ca\(^{2+}\)-dependent membrane binding.

It is also intriguing that this loop is highly flexible in solu-
tion, yet it is likely to have a similar rigid structure in both the crystal and membrane-bound forms. In the annexin B12 x-ray crystal structure, this loop is involved in multiple interactions with neighboring protein molecules and has relatively low thermal factors (7). It seems that these interactions in the crystal “freeze out” the same conformation that this dynamic loop adopts when it interacts with the membrane.

In addition to the strong reduction in the dynamics of the interhelical loop region, we also observed significant changes in the overall mobility at most sites in helix D (Figs. 2 and 3). This effect is weak in the N-terminal region of the helix but very pronounced in the C-terminal region, which is the membrane-proximal region of helix D (Fig. 5). These data suggest that the immobilization of the membrane binding loop is at least one of the factors that constrain the motion of helix D.

In contrast to helix D, much more subtle changes were observed in helix E (Fig. 5). Here, EPR spectral changes were largely confined to sites located on the outside surface. Upon membrane interaction, these sites exhibit a somewhat reduced mobility (Figs. 2 and 3). We interpret this Ca$^{2+}$- and membrane-dependent mobility change to be the consequence of quaternary contacts made upon trimer formation (Fig. 1, bottom).

The interaction of annexin B12 with bilayers is a highly cooperative process that clearly involves multiple interactions with perhaps as many as 12 Ca$^{2+}$ ions per annexin B12 monomer (or up to 36 per trimer), each of which is individually of low affinity (24). Each Ca$^{2+}$ ion is likely to be coordinated by the phosphoryl oxygen from bilayer phospholipid and thus form a “Ca$^{2+}$ bridge” between annexins and the bilayers (8). We have previously noted that the spacing between the Ca$^{2+}$ binding sites and the phospholipid head group regions seems to be complementary (24). One might therefore expect that the complementary spacing of the head groups in bilayers may be essential for protein-lipid interaction. In support of this idea, we saw no changes in mobility of 144R1 when it was incubated in solution with high concentrations of Ca$^{2+}$ (25 mM) and glycerophosphoserine (50 mM), an analogue of the polar head group of phosphatidylserine (data not shown), even though 144R1 was strongly mobilized by Ca$^{2+}$-dependent interaction with bilayers (Fig. 2 and 3). Thus, the physical state of phospholipid membranes clearly plays a critical role in annexin B12 binding.

It should also be pointed out that, based on the crystal structure, the carboxylate of glu142 provides one of the ligands for a type II Ca$^{2+}$ site in the second repeat of annexin B12 (7). Thus, replacement of the native Glu side chain with a nitrooxide could reduce the affinity of this Ca$^{2+}$ binding site or possibly disrupt it. Nonetheless, 142R1 still underwent significant immobilization upon membrane binding (Fig. 2). Considering the large number of Ca$^{2+}$ bound per annexin molecule, it is possible that other sites of annexin B12 were sufficient to cause local immobilization of lipid and protein even in the presence of the mutated type II site.

Molecular motions in a phospholipid bilayer occur over a wide time range. For example, the trans-gauche isomerizations of the hydrocarbon chains occur on a time scale of $10^{-8}$ to $10^{-10}$ s, and, together with the axial diffusion of individual lipid molecules on a time scale about an order of magnitude slower, these motions give rise to liquid-like properties of the bilayer interior. These motions are on the order of the EPR time scale, and spin labels on the lipid chains near the bilayer center have high mobility (25). However, the head group region is more ordered and is characterized primarily by rotation of the lipid about the long axis ($10^{-9}$ to $10^{-10}$), local fluctuations in the head group moiety, and lateral diffusion. Lateral diffusion can be characterized by the lifetime of a lipid at a particular position, before it exchanges with its neighbors. This is on the order of $10^{-7}$ s, very slow on the EPR time scale (26). For a protein-bound spin label, such as 144R1 of annexin B12 located in the head group region of the bilayer, it is thus likely that axial rotational diffusion and local fluctuations will determine the nitroxide motion. These motions are sufficiently rapid to support an intermediate mobility of the nitroxide (correlation times of 1–10 ns). Indeed, R1 side chains on the surface of bacteriorhodopsin, facing the headgroups of the bilayer, have intermediate mobility (15) much higher than that observed for 144R1 on annexin B12 bound to bilayers. Relevant to the present work are SDSL studies of the Ca$^{2+}$-dependent binding to phospholipid bilayers of the C2 domains of phospholipase A$_2$ (27) and protein kinase C (28) to phospholipid bilayers. In these studies, R1 was placed at sites within the Ca$^{2+}$-binding loops of these proteins, and some of these sites localized to the head-group region of the bilayer in the bound state. At these sites in the C2 domains, the bilayer caused some decrease in mobility of the nitroxides, but the effects were minor compared with the bilayer-dependent immobilization of 144R1 in annexin B12. Thus, it is likely that the immobilization of 144R1 in annexin B12 after binding to bilayers is a result of interaction of the nitroxide with phospholipid head groups that are strongly immobilized by a type of protein-bilayer interaction that is unique to the annexins. This phenomenon is not specific to bilayers containing phosphatidylserine, because strong immobilization was also observed when phosphatidylserine was replaced with either phosphatidylglycerol or cardiolipin.2 These results further support the notion that the immobilization of 144R1 is not caused by any specific interaction of the R1 side chain with phosphatidylserine but rather is caused by overall restrictions in the lipid mobility. One would therefore expect that the native serine side chain at position 144 would experience immobilization similar to that observed for the nitroxide side chain at this position.

It is possible that the unusual property of annexin-induced immobilization of bilayer headgroups could have implications for the biological function of this family of proteins. Several annexin gene products have been shown to cause pronounced effects on lipid dynamics after Ca$^{2+}$-dependent membrane binding. FRAP experiments have shown that the lateral diffusion rates of phospholipids are reduced by several orders of magnitude by annexin A4 or A5 binding (29, 30). Furthermore, studies of spin-labeled lipids show that annexin A5 has found profound effects on the lipid fluidity gradient (31, 32), which is naturally present in phospholipid bilayers (25). It is interesting that strong reduction in mobility was observed in particular at lipid sites closest to the head group region (31). Thus, it seems that the overall motion of both the lipid head group and the lipid binding sites in the protein are reduced significantly upon Ca$^{2+}$-dependent interaction. We are not aware of any other instance in which the interaction of a protein and membrane has been shown to cause strong immobilization in both the lipid head group and the protein. Ca$^{2+}$-dependent binding of annexin B12 to bilayers is strongly exothermic, with an enthalpy of association of ~75 kcal/mol protein (24), yet this interaction is fully reversible. The overall free energy of the interaction must be significantly reduced by the entropy loss because of reduced mobility of both protein and lipid. These observations raise the possibility that annexin-induced immobilization of phospholipids could have physiologically significant effects on the mobility of other proteins in biological membranes. In support of this idea, recent studies have shown that the diffusion coefficient of the ryanodine receptor is reduced to near zero in planar bilayers in the presence of 1 μM annexin B12 (33).

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2 J. M. Isaas, R. Langen, W. L. Hubbell, and H. T. Haigler, unpublished results.
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