A Helical Hairpin Region of Soluble Annexin B12 Refolds and Forms a Continuous Transmembrane Helix at Mildly Acidic pH*

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Annexins are soluble proteins that are best known for their ability to undergo reversible Ca$^{2+}$-dependent binding to the surface of phospholipid bilayers. Recent studies, however, have shown that annexins also reversibly bind to membranes in a Ca$^{2+}$-independent manner at mildly acidic pH. We investigated the structural changes that occur upon pH-dependent membrane binding by performing a nitroxide scan on the helical hairpin encompassing helices A and B in the fourth repeat of annexin B12. Residues 251–273 of annexin B12 were replaced, one at a time, with cysteine and then labeled with a nitroxide spin label. Electron paramagnetic resonance (EPR) mobility and accessibility analyses of soluble annexin B12 derivatives were in excellent agreement with the known crystal structure of annexin B12. However, EPR studies of annexin B12 derivatives bound to membranes at pH 4.0 indicated major structural changes in the scanned region. The helix-loop-helix structure present in the soluble protein was converted into a continuous transmembrane α-helix that was exposed to the hydrophobic core of the bilayer on one side and exposed to an aqueous pore on the other side. Asp-264 was on the hydrophobic membrane-exposed face of the amphipathic transmembrane helix, thereby suggesting that protonation of its carboxylate group stabilized the transmembrane form. Inspection of the amino acid sequence of annexin B12 revealed several other helical hairpin regions that might refold and form continuous amphipathic transmembrane helices in response to protonation of Asp or Glu switch residues on or near the hydrophobic face of the helix.

Annexins are a family of structurally conserved proteins that are abundantly expressed in eukaryotes (1). Annexins have been extensively studied as intracellular proteins that localize to the cytosol and undergo reversible binding to intracellular membranes in response to rising Ca$^{2+}$ concentrations (for reviews see Refs. 2 and 3). However, a more limited number of recent studies showed that annexins also associated with membranes by a Ca$^{2+}$-independent mechanism (4–11). Changes in the expression and localization pattern of different annexins have been implicated in several pathological conditions, including cancer and cardiovascular disease (12, 13). Although the precise physiological functions of annexins are not yet fully understood, it is thought that annexins are likely to be involved in membrane-related events such as vesicle trafficking, membrane domain organization, membrane fusion, and cell signaling (2, 14). Because annexins are likely to exert their biological functions while associated with membranes, a molecular understanding of these membrane interactions is important. Structural information on membrane-bound annexins is limited, but high-resolution x-ray crystal structures are available for the soluble forms of numerous annexins.

Annexins have a non-conserved N-terminal domain and a structurally conserved core domain that contains the Ca$^{2+}$-binding sites (1). The core domain usually consists of four repeats that share moderate sequence identity and are ~70 amino acids in length. The crystal structures of the core domains of different annexin gene products are nearly superimposable and show that each of the four repeats contains five α-helices, labeled A–E, in the annexin B12 structure depicted in Fig. 1 (15). Four of these helices form a bundle with the C-helix sitting on top. The core of each bundle contains mainly hydrophobic residues, whereas the surface of the protein and the interfaces between the repeats are mainly hydrophilic. The loop regions between the A-B and D-E helices contain Ca$^{2+}$-binding sites that mediate protein binding to phospholipid bilayers (1). Site-directed spin labeling studies of soluble and Ca$^{2+}$-dependent membrane-bound annexin B12 showed that both forms had structures that were very similar to the x-ray crystal structure (16, 17). Electron microscopy (18, 19) and atomic force microscopy (20, 21) images of annexins bound to the surface of bilayers in the presence of Ca$^{2+}$ also showed that the membrane-bound proteins had the same general shape as observed in x-ray crystals.

A number of annexins have been shown to undergo Ca$^{2+}$-independent association with liposomes and biological membranes at mildly acidic pH (4, 6–11). The exact pH required for membrane interaction varies among the different annexins and is strongly modulated by phospholipid composition (6). Biochemical studies on annexins A5 and B12 demonstrated that acidic pH increases the overall hydrophobicity of these proteins and promotes membrane insertion (7, 8). These conclusions are further supported by the observation that acidic conditions can increase the hydrophobicity of matrix vesicle annexins, which were found to selectively partition into lipophilic organic solvents at acidic, but not at neutral, pH (22). Single-channel conductance showed that annexins A5 and B12 mediated ion flux across phospholipid bilayers at mildly acidic pH (8). Although the physiological significance of the channel-like activity is not yet known, structural models of Ca$^{2+}$-independent binding of annexins to membranes at mildly acidic pH should accommodate this in vitro observation.

Site-directed spin labeling studies of annexin B12 showed that it underwent global refolding upon Ca$^{2+}$-independent association with membranes at mildly acidic pH (6). A nitroxide scan of the region encompassing the D helix-loop-E helix region of repeat II of annexin B12 (see Fig. 1) provided insights into the mechanism of refolding (11).
Acid-induced membrane binding caused this helical hairpin to refold and form a single continuous transmembrane helix. This transmembrane helix was found to be asymmetrically solvated, with one side exposed to the lipid bilayer and the other side exposed to an aqueous cavity, a structure that would be expected to have channel-like activity (11). This structural reorganization was mediated by an inside-out refolding that brings hydrophobic residues buried in the core of the crystal structure into contact with the hydrophobic lipid bilayer. Protonation of two Glu residues near the hydrophobic face of the transmembrane helix was speculated to be the pH-sensitive event that induced refolding and membrane insertion (11). The transmembrane nature of the amphipathic helix identified by the nitroxide scan of the D-E helix region in the second repeat of annexin B12 was confirmed by a separate experimental approach that used fluorescence spectroscopy (23).

The structural model proposed to explain the asymmetric solvation of the transmembrane helix described above requires additional amphipathic transmembrane helices to form the aqueous pore. Because the membrane-inserted form of annexin B12 at mildly acidic pH is monomeric (24), multiple transmembrane regions are likely to be present in the same protein molecule. An inspection of the amino acid sequence of annexin B12 reveals that seven of the eight helical hairpin regions on the convex face of the protein are well suited to form transmembrane amphipathic helices with Glu or Asp residues either exposed to either aqueous solution or to lipid in the membrane-inserted form at pH 4.0 (determined by optical values, see "Results") are indicated by red or yellow, respectively. Residues in the 251–273 scan that had intermediate optical values are shown in white. Residue 257, shown in gray, was not tested. B, the upper panel shows a side view (rotated 90° around the x axis relative to A) of the crystal structure of the soluble form of annexin B12. The D-E helix region in repeat II is highlighted in dark blue and the A-B helix region in repeat IV is highlighted in green and yellow (the yellow faces of the helices correspond to the yellow spheres in A). Both helical hairpins refold and form continuous transmembrane amphipathic helices upon Ca<sup>2+</sup>-independent binding to membranes at mildly acidic pH (bottom panel). In the bottom panel, the lipid-exposed residues (yellow) that were analyzed in the current study are positioned according to their immersion depths (see "Results"). The gray cylinders represent other putative transmembrane helices (see "Discussion"). The number of these putative helices is arbitrary.

**Experimental Procedures**

Protein Expression and Purification—A series of 23 single cysteine mutations was introduced into the appropriate sites in the annexin B12 pSE420-mp33H plasmid (30) by site-directed mutagenesis (QuikChange, Stratagene). All mutants were verified by sequencing. The annexin B12 mutants were expressed in DH5α Escherichia coli and purified by reversible Ca<sup>2+</sup>-dependent binding to phospholipid vesicles followed by size-exclusion chromatography, as previously described (30, 31). The proteins were stored in 20 mM Hepes containing 100 mM NaCl at pH 7.4 (Hepes–NaCl), supplemented with 1 mM dithiothreitol. Final protein concentrations were determined by absorbance at 280 nm and use of the extinction coefficient ε = 12,288 M<sup>−1</sup> cm<sup>−1</sup>

Spin Labeling and EPR Spectroscopy—Immediately prior to spin labeling, dithiothreitol was removed from the buffer by size exclusion chromatography with PD-10 columns (Amersham Biosciences). The

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*The abbreviation used is: NiEDDA, Ni(II) ethylenediaminetriacetic acid.*
pH and Membrane-dependent Refolding of Annexin B12

proteins were eluted with Hepes-NaCl buffer and were concentrated with centrifugal filter devices (Amicon Ultra-4 10,000 MWCO). The introduced cysteines were reacted (~1 h, 22 °C) with a 3-fold molar excess of 1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl methanethiosulfonate. Unreacted spin label was removed with a PD-10 column. R1 labeling did not appear to structurally or functionally alter 22 of the residues, especially residue 255R1.

 RESULTS

 Structural Analysis of Annexin B12 in Solution—To study the conformational changes that occur in annexin B12 upon Ca2+-independent membrane binding at mildly acidic pH, we performed a nitroxide spin labeling experiment. Single Cys residues were engineered into sites 251–253R1, 266R1–273R1 and used to attach the side chain R1 (Fig. 2). All sites except 257 tolerated R1 labeling (see “Experimental Procedures”). The mobility and accessibility of R1 in each of these derivatives were then determined by EPR spectroscopy to obtain structural information for soluble and membrane-bound forms of the protein.

 The EPR spectra of all sites analyzed in aqueous solution at pH 7.4 (Fig. 3A, red traces) were consistent with the crystal structure of annexin B12. For example, the EPR line shapes for residues 258R1–263R1 were very sharp and narrowly spaced, indicative of a high degree of mobility. Such elevated mobility is a characteristic feature of loop or unfolded regions (16, 28) and agrees well with the location of these residues in the loop between the A and B helices in the crystal structure (Fig. 1). The inverse central line width, ΔH1/2, is a measure of R1 mobility with higher values indicating higher mobility (28). A plot of ΔH1/2 as a function of amino acid sequence number clearly illustrates the high mobility associated with the 258–263 loop region (Fig. 3B, red circles). In contrast, spectra for residues 264R1–273R1, which correspond to the B-helix that is largely buried in the core of the protein, exhibited broad lines and increased spectral breadth characteristic of buried and immobilized sites (16, 28) (Fig. 3A, red traces and B, red circles). Note that strongly immobilized spectral components are highlighted by red arrows. Residues 264R1 and 265R1, which correspond to the beginning of the B-helix and face the outer surface of the protein, were more mobile than residues at the center of helix B, which were buried in the center of the protein (Fig. 3A, red and B). In addition to its mobile component, residue 264 exhibited a more immobilized component that could be caused by tertiary contacts with the side chains of residues 302 and 74 (15). In the crystal structure, helix A is mostly buried but is partially exposed to the solvent (Fig. 1). The EPR spectra of residues 251R1–256R1 are consistent with this structure. Strong immobilization was observed at residues that are buried in the crystal structure (Fig. 3A, red, arrows, and B), whereas less immobilization was observed at the surface-exposed residues, especially residue 255R1.

 All of the experiments performed on annexin B12 in solution at pH 7.4, as reported above, were also performed at pH 4.0 in the absence of membranes. In solution, the EPR mobility parameters at pH 4.0 (data not shown) were nearly identical to the results obtained at pH 7.4. The overall structure of the scanned region was essentially the same in solution at neutral and mildly acidic pH. Previous studies of other regions of annexin B12 also indicated that the backbone fold of the protein did not change as a function of pH in the 4.0–7.4 range when the protein was in solution in the absence of phospholipid (6, 11).

 To further characterize the structure of annexin B12 in solution, we determined the local accessibilities of the R1-labeled proteins to collision with the paramagnetic reagents O2 and NiEDDA. The accessibility parameters Π(O2) and Π(NiEDDA) were in-phase throughout the scanned 251–273 region (Fig. 4), as is expected for a soluble protein based on previous studies showing that both reagents collide with residues on the surface of the protein and are excluded from the interior (26). Residues in the loop region of annexin B12 (258R1–263R1) had high accessibility values, whereas buried residues in both helices (251R1–253R1, 266R1–273R1) were inaccessible to both colliders. Sites in the A (254R1, 255R1) and B helices (264R1, 265R1), which are exposed to the surface of the protein in the crystal structure, had intermediate Π(O2) and Π(NiEDDA) values (Fig. 4).

 Overall, there was a high correlation between mobility and accessibility to both O2 and NiEDDA (Figs. 3, A and B, and 4) in the scanned region of annexin B12 in solution. Both mobility and accessibility data were entirely consistent with the x-ray crystal structure of the soluble form of annexin B12.

 Structural Analysis of Annexin B12 Bound to Membranes at Mildly Acidic pH—To investigate structural and dynamic changes that occur upon Ca2+-independent binding to bilayers at mildly acidic pH, we recorded the EPR spectra for R1-labeled annexin B12 derivatives in the presence of large vesicles composed of phosphatidylserine-phosphatidylcholine (2:1 w/w) at pH 4.0. As shown in Fig. 3A, most of the spectra for the membrane-bound form (black traces) were notably different from those of the soluble form (red traces). These changes also were apparent when the mobility parameter, ΔH1/2, was plotted as a function of amino acid sequence number (Fig. 3B, black circles). The most pro-

FIGURE 2. Structure of the R1 side chain. Sulphhydryl groups introduced into annexin B12 were reacted with (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate to produce the paramagnetic R1 nitroxide side chain.
nounced changes were observed for sites in the highly dynamic loop region (residues 258R1–263R1). In the membrane-bound form, the EPR spectra of all loop sites became much broader and lower in amplitude, indicating a significant reduction in motion. In the membrane-bound form, the spectra of 258R1–263R1 are not consistent with a loop structure and indicate the formation of a folded structure in this region (Fig. 3).

The EPR spectra (Fig. 3A) for sites in the regions corresponding to helix A and helix B were consistent with an ordered structure in the membrane-bound form. Nonetheless, a comparison of the spectra for the soluble and the membrane-bound forms revealed conformational changes at most R1-labeled sites. Sites with low mobility in solution that were buried in the crystal structure showed significant increases in mobility in the membrane-bound form (Fig. 3, A and B). These changes indicated that binding to membranes induced structural changes that disrupted the specific tight packing interactions in the core of the helical bundle in the fourth repeat of the protein. Also note that 255R1, which had intermediate mobility and was exposed to the surface of the protein in solution, became less mobile upon membrane binding (Fig. 3, A and B). Thus, an analysis of all the R1-labeled sites in this nitroxide scan indicated that both the helical and loop regions underwent significant conformational changes in response to binding to membranes at mildly acidic pH. The plot of $\Delta H_0^{-1}$ as a function of sequence revealed intermediate mobility parameters that did not show any obvious periodic changes through this region (Fig. 3B, black circles). The individual spectra of the membrane-bound derivatives do not contain well resolved outer hyperfine extrema (Fig. 3A, black traces), indicating the lack of strong packing interactions.

To obtain additional structural information and to determine whether sites in the scanned region were exposed to the hydrophobic core of membranes following binding at mildly acidic pH, we determined the accessibility of R1-labeled derivatives to O2 and NiEDDA (O2) and NiEDDA (NiEDDA), respectively. As shown in Fig. 5A, the O2 and NiEDDA accessibility data for the membrane-bound annexin B12 derivatives were quite different from those obtained for the soluble form. Although the accessibility data of the soluble protein clearly revealed three separate regions (helix A, the loop, and helix B), the O2 and NiEDDA accessibilities of membrane-bound annexin exhibited a
continuous periodic oscillation throughout the entire scanned region. The underlying periodicity of the latter was remarkably regular, with local maxima and local minima spaced ~3–4 amino acids apart. Such periodicities are highly characteristic of \( \alpha \)-helical structures, which contain an average of 3.6 amino acids/turn.

Another striking difference between the soluble and membrane-bound forms was that the \( O_2 \) and NiEDDA accessibilities were out of phase in the membrane-bound form, while they were in-phase in the soluble protein. Extensive previous studies have shown that out of phase periodicity is caused by a differential effect of membranes on \( O_2 \) and NiEDDA accessibilities (26).

Two structural models are consistent with an asymmetrically solvated \( \alpha \)-helix of the type identified above. The helix could be parallel to the membrane in the interfacial region of the membrane or it could be transmembrane, lining a water-filled pore (see “Discussion”). Both types of structures have been reported for other proteins (26, 29, 33, 34). One can distinguish between these two models by determining the immersion depth of the lipid-exposed residues (26). Although membrane-exposed residues in a peripherally bound helix will be at comparable immersion depth, the immersion depth of lipid-exposed residues in a transmembrane helix will increase linearly toward the center.

The immersion depth of the residues with maximal lipid exposure in the scanned region of annexin B12 was determined from the parameter \( \Phi \) and a calibration curve as described under “Experimental Procedures.” As shown in Fig. 6, the immersion depths increased linearly on either side of 264R1, which, at an immersion depth of ~20 Å, represented the deepest residue. These data were inconsistent with a peripherally bound \( \alpha \)-helix but were in excellent agreement with the formation of a transmembrane helix.
DISCUSSION

Previous studies (30, 31) showed that annexin B12 exists in three forms: a soluble monomer, a Ca\(^{2+}\)-dependent peripheral membrane-bound trimer (31), and a novel Ca\(^{2+}\)-independent transmembrane monomer (8, 11, 23, 24). These three forms undergo reversible interconversion, with the equilibrium modulated by phospholipid, Ca\(^{2+}\), and H\(^+\). The primary goal of the current study was to use a site-directed spin labeling approach to compare the structure of the A helix-loop-B helix region of the fourth repeat of annexin B12 in the soluble and Ca\(^{2+}\)-independent membrane-inserted forms. In solution, the EPR mobility (Fig. 3, A and B) and accessibility (Fig. 4) parameters for the scanned region were consistent with the crystal structure of the protein. Other studies showed that this region had a structure similar to the crystal form of annexin B12 in the Ca\(^{2+}\)-dependent membrane-bound form.\(^5\)

However, significant changes in both mobility (Fig. 3, A and B) and accessibility (Fig. 5) were observed at most sites in this region when these EPR parameters were measured following pH-induced association with membranes. The helix-loop-helix structure observed in solution was converted into a continuous α-helix in the membrane-bound state. Accessibility data clearly showed that the membrane-associated helical structure was asymmetrically solvated with one side exposed to the lipid and the other side exposed to an aqueous environment (Fig. 5). The depth profile of the lipid-exposed residues showed that the helix was transmembrane (Fig. 6). Arg-274 is located one residue beyond this transmembrane helix, and this positively charged residue may stabilize the proposed structure by interacting with the negatively charged head groups of the phospholipid bilayer.

A structural model that is consistent with these data is presented in Fig. 1B. In this model, the hydrophilic face of the scanned α-helix faces an aqueous pore formed by several other amphipathic transmembrane helices (further discussed below). This pore structure may mediate the in vitro ion channel-like activity of annexin B12 (8). The hydrophobic face of the transmembrane helix faces the core of the bilayer. Most of the residues on the lipid-facing side of the transmembrane α-helix are hydrophobic amino acids that were buried in the core of the crystal structure of soluble annexin B12 (Figs. 1A and 5C). However, Asp-264 is also located on the center of the hydrophobic face of the helix (Fig. 5C) at the site that is most deeply buried in the bilayer (Fig. 6). The interaction of charged carboxylate Asp side chains with the hydrophobic core of bilayers is very energetically unfavorable, but uncharged protonated side chains (as expected at pH 4) can be embedded in bilayers without penalty (35). It is interesting to note that Asp-264 is likely to be a Ca\(^{2+}\)-coordination site that participates in Ca\(^{2+}\)-dependent binding of annexin B12 to the surface of bilayers. Thus, a switch between the interaction of Asp-264 with Ca\(^{2+}\) and H\(^+\) may be a trigger for refolding and membrane insertion of this region of annexin B12.

There are a number of structural similarities and a few differences between the pH-induced membrane insertions of the D-E hairpin that was previously reported (11) and the A-B hairpin described herein. The common elements are that both helical hairpins undergo membrane insertion and pH\(^+\)-induced refolding to form continuous transmembrane amphipathic helices with Glu or Asp residues on or near the hydrophobic faces. Both transmembrane helices are ~20 amino acids long and are flanked by positively charged amino acids that may interact favorably with the negatively charged head groups of the bilayer phospholipids. Both transmembrane helices are amphipathic and are formed by inside-out refolding of helices in the soluble protein. In the soluble form of annexin B12, the hydrophilic face of the A, D, and E helices largely face the solvent-exposed external surface of the protein. In contrast, the hydrophilic face of the B-helix is buried in the center of the protein. It is unusual for soluble proteins to pack hydrophilic side chains in their core. In annexin B12, this feature is accomplished by a mechanism in which the buried hydrophilic face of the B-helix interacts with hydrophilic residues across the interface of different repeat domains in the center of the protein.

A major difference between the helical hairpins that have been scanned in annexin B12 is that the loop between the D and E helices contains three residues, whereas the loop between the A and B helices contains six residues. These loops refold and contribute approximately one or two turns, respectively, to the transmembrane helix. In both cases, the length of the loop is such that the hydrophobic faces of the original helix-loop-helix regions line up on the same side of the transmembrane helix.

The model shown in Fig. 1B depicts an aqueous pore formed by several amphipathic helices. Because the Ca\(^{2+}\)-independent membrane-inserted form of annexin B12 is a monomer (24), several other regions capable of forming transmembrane helices are thought to be present within the amino acid sequence of this protein. An inspection of the amino acid sequence of annexin B12 reveals five additional helical hairpins with structural similarities to the transmembrane helices formed by the A-B helical hairpins in this study and the D-E region in the previous study (11). These transmembrane helices (five putative plus the two subject to nitroxide scans) correspond to seven of the eight A-B and D-E helical hairpins on the convex face of annexin B12. The A-B helical hairpin of repeat III was not included as a putative transmembrane helix because it did not contain a region that could form a continuous α-helix, perhaps because it contains one more amino acid in its loop than are present in the other three A-B loops. The seven putative transmembrane helices are reasonably amphipathic and also harbor Glu and Asp residues on or near the hydrophobic face. Each of these helices also contains ~20 amino acids and is therefore of sufficient length to span the bilayers. In addition, each helix is flanked by either positively charged residues or good turn-forming residues, a feature commonly observed in transmembrane helices. Thus, these regions also might be able to refold and form continuous transmembrane helices as indicated by the gray cylinders shown in Fig. 1B. Future studies are needed to evaluate the validity of these additional transmembrane regions of annexin B12, and to determine the physiological significance of Ca\(^{2+}\)-dependent membrane insertion of this protein.

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