Insertion intermediate of annexin B12 is prone to aggregation on membrane interfaces

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Annexin B12 (ANX) is known to insert across the lipid bilayer at acidic pH in the absence of Ca^{2+} and to form a pore-like structure consisting of several transmembrane helices, most of which are unknown. Our previous studies demonstrate that the insertion proceeds via an interfacial refolded intermediate state, which can be stabilized by anionic lipids. Energy transfer measurements in a mixture of donor- and acceptor-labeled ANX indicate that this interfacial intermediate, unlike the final transmembrane conformation, is prone to aggregation. Such aggregation of a non-inserted ANX may have implications for a possible general mechanism of misfolding of membrane proteins.

Keywords: membrane protein, transmembrane helix, folding/insertion intermediate, lipid bilayer topology, circular dichroism, FRET.

Annexins are a structurally conserved family of proteins implicated in a variety of membrane-related functions, including vesicular trafficking, membrane fusion, and ion-channel formation [1–3]. They are also associated with several diseases known as «annexinopathies» [4]. High-resolution crystal structures of the soluble forms of several different annexins, including annexin B12 (ANX) [5], reveal a common fold. Besides the well-documented Ca^{2+}-dependent binding to membrane interfaces, shared by most annexins, ANX has been demonstrated to insert in a transmembrane (TM) conformation at acidic pH [6, 7]. Recently we have demonstrated that this transition is reversible [8], which made ANX an attractive model for folding studies. Previously we have identified an interfacial insertion intermediate, stabilized by electrostatic interactions on the membrane interface [7]. The application of the FRET (Förster resonance energy transfer)-based methodology indicated that the insertion intermediate, but not the final TM inserted form, is prone to aggregation on membrane interfaces.

Materials and Methods. *Materials*. POPC (palmitoyloleoylphosphatidylcholine), POPG (palmitoyloleoylphosphatidylglycerol), and lysoPE were purchased from Avanti Polar Lipids (Alabaster, AL). Alexa-532 C5 meleimide and Alexa-647 C2 maleimide were purchased from Molecular Probes (Eugene, OR). A 10 mM sodium acetate buffer with pH 4.5 containing 50 mM KCl and 1 mM EDTA was used.
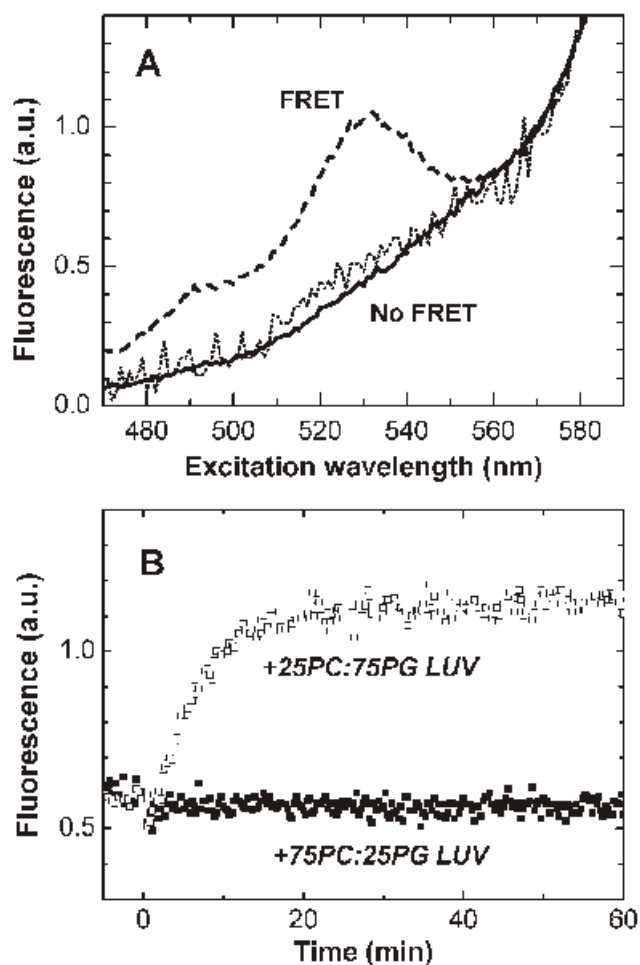
Fig. 1. FRET experiment establishing the aggregation state of ANX along the pH-triggered insertion pathway: *A* – excitation spectra are measured for a 1:1 mixture of ANX mutant K132C labeled with Alexa-532 (donor) or Alexa-647 (acceptor) as described in [8] (all samples contained a total of 0.4 mM lipid; aggregation-related FRET signal is observed for 1 µM ANX in vesicles of high anionic lipid content 25PC:75PG (dashed), which is known to stabilize interfacial intermediate state [7]; a 10-fold reduction in ANX concentration till 0.1 µM abolishes aggregation (dotted line); no aggregation-related FRET was observed for 75PC:25PG lipid composition (known to promote insertion [7]), even at 1 µM ANX concentration (solid line); *B* – the kinetics of fluorescence change measured with 530 nm excitation is shown in the insert (membrane insertion was initiated at zero time).

Single-cysteine mutant of ANX K132C was labeled with Alexa dyes as described previously [8].

Sample preparation. Large unilamellar vesicles (LUV) of diameter 0.1 µm were prepared by extrusion [9] from either 3:1 or 1:3 molar mixtures of POPC and POPG. ANX insertion was initiated by addition of appropriate amounts of LUV into protein solution.

Steady-state fluorescence. Fluorescence was measured using an SLM 8100 steady-state fluorescence spectrometer (Jobin Yvon, Edison, NJ, former SLM/AMINCO, Urbana, IL) equipped with double-grating excitation and single-grating emission monochromators as described previously [8]. The measurements were made in 4 × 10 mm cuvettes oriented with the long axis parallel to the excitation beam. Temperature was maintained at 25 °C using a circulating water bath. Cross-orientation of polarizers was used (excitation polarization set to vertical, emission polarization set to horizontal) in order to minimize the scattering contribution from vesicles, eliminate spectral polarization effects in monochromator transmittance and enhance the sensitivity of FRET measurements [8]. Fluorescence excitation spectra of 1:1 mixture of donor- and acceptor-labeled annexins were obtained by averaging 5–10 scans collected over a 470–660 nm range using 1 nm steps. The emission monochromator was set at 680 nm. Excitation slits were 8 nm and emission slits were 16 nm. Kinetic measurements were obtained with a 15 sec resolution after hand mixing of LUV into protein sample using 530 nm excitation and 680 nm registration.

CD (circular dichroism) measurements. CD measurements were performed using Jasco-720 spectropolarimeter (Japan Spectroscopic Company, Tokyo). Normally, 70 scans were recorded between 190 and 260 nm with a 1 nm step at +25 °C, using a 1 mm optical path cuvette. Samples for CD measurements contained 1 µM ANX and 0.4 mM LUV, when present. All spectra were corrected for background by subtracting a vesicle-only spectrum measured with an appropriate concentration of LUV in buffer, without the ANX.

Results and Discussion. The final inserted state of ANX is an aqueous pore, comprised of several TM helices [6, 7]. Previously we have demonstrated that the efficiency of insertion decreased with an increase of the fraction of anionic lipids, which stabilize the intermediate refolded state in its interfacial conformation [7]. Here we examine the aggregation properties of this interfacial intermediate.

Recently we have demonstrated via a FRET approach that ANX is a monomer in the inserted TM
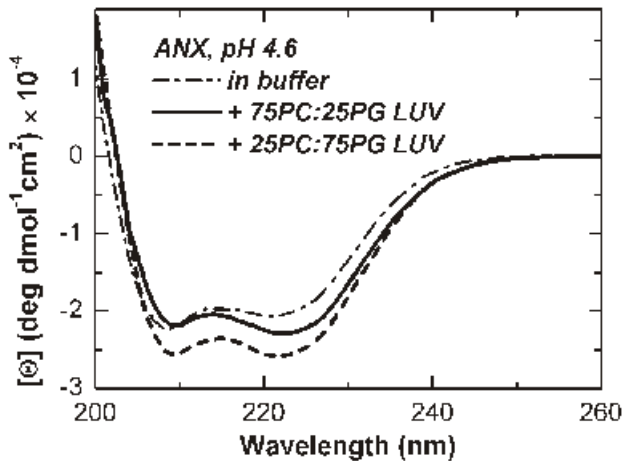
Fig. 2. CD measurements of ANX in buffer and in the presence of various LUV. While all curves have a pronounced helical character, the exact secondary structures of the samples corresponding to various states (*W*-state: dash-dotted line, transmembrane *T*-state: solid line and aggregated interfacial *A*-state: dashed line) are different. Due to relatively low sensitivity of the CD measurements (as compared to fluorescence), no reliable signal can be collected under dilute conditions corresponding to non-aggregated *I*-state.

state [8]. When the same methodology is applied to the intermediate state, however, a clear FRET signature of aggregation is apparent (25PC:75PG sample – mixtures of POPC and POPG that contain a molar percentage of corresponding lipid specified by the number, Fig. 1 *A*, dashed line). No aggregation was observed when different lipid composition was used (75PC:25PG sample, solid line) or when ANX concentration was reduced 10-fold (25PC:75PG sample, dotted line). ANX and lipid concentrations in the letter dilute sample correspond to those used in the fluorescence lifetime topology experiment [7]. Thus the aggregation is not the reason for hindered insertion, but rather accumulation of the non-inserted intermediate can lead to its aggregation. The kinetic change in FRET-associated signal (Fig. 1, *B*) is over after 20 minutes, which is faster than the insertion time for this lipid composition (Posokhov, Rodnin, Lu, Ladokhin, Biochemistry, 2008 in press).
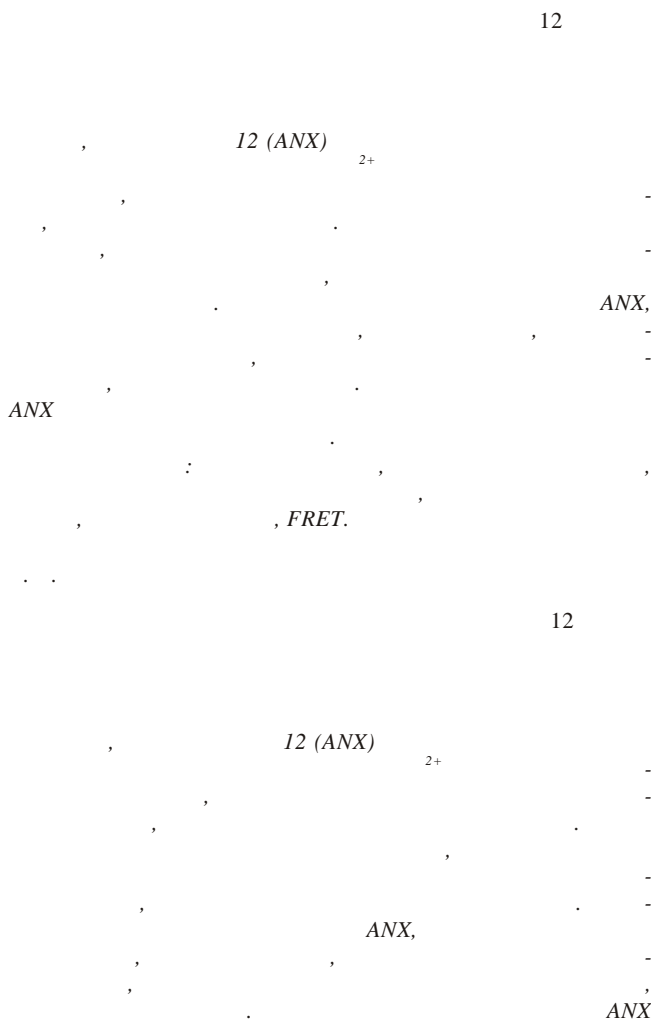
We have examined the secondary structure of the ANX in various LUV using CD spectroscopy (Fig. 2). All samples gave a characteristic double negative peak of -helix dominated structure, with the sample corresponding to the aggregated state (25PC:75PG, Fig. 2, dashed line) having stronger ellipticity and,

hence, higher helical content. This is not surprising since membrane interfacial region is known to promote helix formation via partitioning-folding coupling [10, 11]. We did not consider attempting a quantitative estimate of the secondary structure, because the absence of the appropriate base spectra for membrane proteins in general (let alone, for interfacial (IF), , and TM helixes separately) makes such analysis highly speculative. In addition, high scattering at wavelengths shorter than 200 nm prevented accurate acquisition of data in this region, crucial for quantitative analysis of secondary structure. Nevertheless, we point out that the three curves, corresponding to soluble *W*-state (dash-dotted line), transmembrane *T*-state (solid) and aggregated interfacial *A*-state (dashed) do not share an isodichroic point. This indicates that the underlying structure of either of them is unique and can not be presented as a weighted sum of the other two.

Perspectives. One of the reasons that studies of folding and stability of membrane proteins lag far behind those of their soluble counterparts is a shortage of appropriate experimental models of folding, and what perhaps is even more challenging, of misfolding. Recently we have demonstrated that ANX is a useful model, allowing the measurements of various thermodynamic parameters of membrane insertion/folding reaction (Posokhov, Rodnin, Lu, Ladokhin, Biochemistry, 2008 in press). Evidence presented here suggests that ANX, when bound to membranes of appropriate lipid composition, can also serve as a model for a misfolding process in which hindered insertion leads to aggregation on membrane interfaces. In the cell, a mis-folded(-inserted) state of constitutive membrane proteins is believed to be quickly disposed of via a tightly set protein degradation machinery [12, 13]. Moreover, even a large fraction of the inserted protein is degraded (e. g., ~50 % for CFTR (cystic fibrosis transmembrane conductance regulator) [12]) in order to minimize the risk of accumulating any mis-inserted protein. Our evidence for the interfacial state of ANX being prone to aggregation sheds some light on the possible reasons why this may be the case. If aggregation on membrane interfaces is also shared by mis-inserted constitutive proteins, the cell must act quickly to dispose of them or else deal with much tougher aggregates. We will follow the example of

Sanders and Myers, who used the following quote to draw attention to the danger facing folding membrane protein (Charles Spurgeon, sermon at Newington, 1889, quoted after [13]): «Where there is danger there should be prudent haste. Quick! Pilgrim, be quick and tarry not in the place of danger» … or you might end up aggregated on membrane interfaces.

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REFERENCES

1. *Swairjo M. A., Seaton B. A*. Annexin structure and membrane interactions: A molecular perspective // Annu. Rev. Biophys. Biomol. Struct.–1994.–**23**.–P. 193–213.

2. *Moss S. E., Morgan R. O*. The annexins // Genome Biol.–2004.–**5**.–P. 219–219.8.

3. *Rescher U., Gerke V*. Annexins – unique membrane binding proteins with diverse functions // J. Cell Sci.–2004.–**117**.–P. 2631–2639.

4. *Rand J. H*. «Annexinopathies» – A new class of diseases // New Engl. J. Med.–1999.–**340**.–P. 1035–1036.

5. *Luecke H., Chang B. T., Mailliard W. S., Schlaepfer D. D., Haigler H. T*. Crystal structure of the annexin XII hexamer and implications for bilayer insertion // Nature.–1995.–**378**–P. 512–515.

6. *Langen R., Isas J. M., Hubbell W. L., Haigler H. T*. A transmembrane form of annexin XII detected by site-directed spin labeling // Proc. Nat. Acad. Sci. USA.–1998.–**95**.–P. 14060–14065.

7. *Ladokhin A. S., Isas J. M., Haigler H. T., White S. H*. Determining the membrane topology of proteins: Insertion pathway of a transmembrane helix of annexin 12 // Biochemistry.–2002.–**41**.–P. 13617–13626.

8. *Ladokhin A. S., Haigler H. T*. Reversible transition between the surface trimer and membrane-inserted monomer of annexin 12 // Biochemistry.–2005.–**44**.–P. 3402–3409.

9. *Mayer L. D., Hope M. J., Cullis P. R*. Vesicles of variable sizes produced by a rapid extrusion procedure // Biochim. et Biophys. Acta.–1986.–**858**.–P. 161–168.

10. *Ladokhin A. S., White S. H*. Folding of amphipathic -helices on membranes: Energetics of helix formation by melittin // J. Mol. Biol.–1999.–**285**.–P. 1363–1369.

11. *Fernandez-Vidal M., Jayasinghe S., Ladokhin A. S., White S. H*. Folding amphipathic helices into membranes: amphiphilicity trumps hydrophobicity // J. Mol. Biol.–2007.–**370**.–P. 459–470.

12. *Sanders C. R., Nagy J. K*. Misfolding of membrane proteins in health and disease: The lady or the tiger? // Curr. Opin. Struct. Biol.–2000.–**10**.–P. 438–442.

13. *Sanders C. R., Myers J. K*. Disease-related misassembly of membrane proteins // Annu. Rev. Biophys. Biomol. Struct.–2004.–**33**.–P. 25–51.

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