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# Binding to Phosphatidyl Serine Membranes Causes a Conformational Change in the Concave Face of Annexin I

Milton de la Fuente and Carmen Gloria Ossa
Departamento de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Santiago, Chile

ABSTRACT Recent studies have revealed that binding of annexin I to phospholipids induces the formation of a second phospholipid binding site. It is shown that the *N* terminus on the concave side of membrane-bound annexin I is cleaved much faster by trypsin or cathepsin than the *N* terminus of the free protein. The reactivity of the unique disulfide bond located near the concave face was similarly increased by membrane binding. These results demonstrate that $Ca^{2+}$-dependent membrane binding induces a conformational change on the concave side of the annexin I molecule and support the notion that this face of the molecule may contribute to the formation of the secondary membrane-binding site.

## INTRODUCTION

Annexins are a family of ubiquitous proteins that bind to acidic membrane phospholipids in the presence of $Ca^{2+}$ (Klee, 1988; Raynal and Pollard, 1994). They possess a core structure composed of four or eight repetitions of a conserved 70 amino acid sequence and a variable *N* terminus. Annexins have been implied in a number of processes, including exocytosis, regulation of inflamation, cell growth, cation channel activity, and inhibition of coagulation (Tait et al., 1988; Peers and Flower, 1990; Schlaepfer and Haigler, 1990; Zaks and Creutz, 1990; Raynal and Pollard, 1994).

Annexin I can aggregate secretion granules and liposomes in a $Ca^{2+}$-dependent manner, which has led to the proposal that this protein plays a role in calcium-regulated exocytosis (Lee et al., 1991; Francis et al., 1992; Meers et al., 1993; Ohnishi et al., 1995). The protein has a slightly convex face, which exposes highly conserved residues that bind $Ca^{2+}$ ions, and an opposed slightly concave face, which includes the nonconserved *N* terminus (Weng et al., 1993). The convex domain is proposed to be the $Ca^{2+}$-dependent acidic phospholipid binding domain. Annexin I in solution does not bind to neutral phospholipids such as PC, nor does it bind to acidic phospholipids in the absence of $Ca^{2+}$. However, in a recent study of the mechanism of aggregation of liposomes by annexin I we found that membrane-bound annexin I rapidly aggregated PS or PC liposomes, and this process appeared to be $Ca^{2+}$ independent (de la Fuente and Parra, 1995). This implies that the membrane-bound protein molecules displays a second membrane-binding site, which is probably formed by a conformational transition induced by $Ca^{2+}$-dependent membrane binding. The results also showed that annexin I molecules could simultaneously bind to two vesicles, suggesting that the two membrane-binding sites are on opposite sides of the protein molecule (i.e., the concave face of the protein might be related to the secondary site). Accordingly, the objective of this study was to investigate whether $Ca^{2+}$-dependent membrane binding induces a conformational change in the concave face of annexin I.

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Address reprint requests to Dr. Milton de la Fuente, Departamento Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Casilla 70005, Correo 7, Santiago, Chile. Tel.: 56-2-678-6202; Fax: 56-2-777-6916; E-mail: mdelafue@machi.med.uchile.cl.

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## MATERIALS AND METHODS

Lipids were obtained from Avanti Polar Lipids (Pelham, AL). Anti-annexin I antibody was from Zymed Labs (South San Francisco, CA). TPCK-treated trypsin and cathepsin D were from Sigma Chemical Co. (St. Louis, MO). The rest of the reagents were of analytical grade. Large unilamellar liposomes were prepared by extrusion as described (de la Fuente and Parra, 1995). Annexin I was purified from human placenta as described (de la Fuente and Parra, 1995). Aggregation was carried out in 0.1 M NaCl, 10 mM Hepes, pH 7.4, followed by measurement of absorbancy at 350 nm as described (de la Fuente and Parra, 1995). Rates of aggregation were computed from initial slopes of absorbance-versus-time plots and are expressed as change in absorbance units per minute. All proteolytic degradations and kinetic experiments were carried out at 25°C.

In all the experiments the reactivity of the membrane-bound annexin I was compared with the reactivity of the free protein. Annexin I was bound to PS or PE:PS (3:1) liposomes under conditions ensuring that no aggregation occurred in incubations of at least 30 min (a low protein/lipid ratio and a nominal $Ca^{2+}$ concentration of 50 $\mu$M (de la Fuente and Parra, 1995). Absence of aggregation was confirmed by absorbance measurements. Full binding of annexin I was verified by ultracentrifugation and measurement of the protein bound to the liposomes. Electrophoresis were carried out by use of 12% gels as described by Laemmli (1970).

## RESULTS

One relevant feature of the structure of the concave side of the protein is the presence of the *N* terminus. This sequence is much more susceptible to proteases than is the conserved core, both in situ (De et al., 1986; Haigler et al., 1987; Chuah and Pallen, 1989) and in vitro (Schlaepfer and Haigler, 1987; Ando et al., 1989; Wang and Creutz, 1994). Cathepsin D, calpain, trypsin, and plasmin each rapidly cleave only one particular bond in the *N* terminus, with no further degradation of the annexin core (Schlaepfer and Haigler, 1987; Ando et al., 1989; Wang and Creutz, 1994),

at least within the time required for the completion of the first cleavage. This simple pattern of proteolysis prompted us to attempt to detect conformational changes in the *N* terminal by measuring rates of proteolysis. We used trypsin, which under controlled conditions cleaves annexin I to [des 1–26] annexin I (Haigler et al., 1987), and cathepsin D, which produces [des 1–12] annexin I (Ando et al., 1989). The upper gel in Fig. 1 shows the trypsinolysis of annexin I (*I*), both bound to PS liposomes and in solution, forming [des 1–26] annexin I (*D*). Proteolysis of the PS-bound annexin I was almost complete after a 1-min incubation with trypsin. However, complete cleavage of the free annexin I required ~30 min. The rate of proteolysis was also increased after binding of annexin I to PE:PS (3:1) liposomes (lower gel in Fig. 1). It can be seen that, under the conditions used, trypsinolysis was almost complete after 2 min (*lane 4*), whereas the free protein was essentially intact at the same time. Up to 2 mM $Ca^{2+}$ did not enhance the rate of trypsinolysis of the free annexin I (not shown). Therefore, the acceleration in the rate of proteolysis is due to membrane binding. Additionally, a faint band of a derivative larger than [des 1–26] annexin I was observed at early times (1 min in the upper gel of Fig. 1 and 0.5 and 1 min in the lower gel of Fig. 1). This derivative was not observed in the proteolysis of free annexin I. The only possible point of tryptic cleavage that leads to a derivative that will transform itself in [des 1–26] annexin I is at Lys-9. Thus, we conclude that membrane binding by the convex face of the molecule increases the reactivity of bonds 9 and 26 at the *N* terminus at the opposite side of the molecule. Annexin I remained fully active whether free in solution or membrane bound during the incubations (aggregation assays not shown). This rules out denaturation as a cause for the difference in the reactivity of the free and membrane-bound forms of the protein.

To quantify the difference in reactivity to trypsin we decided to take advantage of the reported changes in $Ca^{2+}$ requirement for vesicular aggregation caused by proteolysis of annexin I. It has been reported that [des 1–26] annexin I required less $Ca^{2+}$ for aggregation of chromaffin granules than did the intact protein, whereas aggregation by [des 1–12] annexin I required more $Ca^{2+}$ (Wang and Creutz, 1994). However, we found the opposite pattern for the aggregation of liposomes. As shown in the upper panel of Fig. 2, [des 1–26] annexin I (■) required more $Ca^{2+}$ for aggregation of PS liposomes than did the intact protein (●). On the other hand, and as shown in the lower panel of Fig. 2, aggregation by [des 1–12] annexin I (■) required less $Ca^{2+}$ than the native protein. The controls in the two panels are not identical because incubation at pH 5, which is required for generation of [des 1–12] annexin I, changed the $Ca^{2+}$ dependency as shown. The reasons for the discrepancy with the chromaffin-granule results are not clear at this time. Nonetheless, the experiments shown in Fig. 2 suggested that the progression of trypsinolysis could be followed from the decrease in the rate of aggregation if it were measured at suboptimal $Ca^{2+}$. Indeed, the aggregation activity (measured at suboptimal $Ca^{2+}$) of both membrane-bound and free annexin I decayed with time on incubation with trypsin (Fig. 3). As expected, aggregation activity remained unchanged in both cases when it was measured at saturating $Ca^{2+}$ (not shown). Measured at subsaturating $Ca^{2+}$, the aggregation activity of membrane-bound annexin I (○ and *inset*) decayed much faster than the free protein (●), in agreement with the results shown in Fig. 1. The curves of Fig. 3 show the fit of the data to an equation assuming a single irreversible transition from a protein of a higher aggregating activity (intact annexin I) to one of a lower activity ([des 1–26] annexin I). Comparison of the apparent rates constants thus obtained (see the caption of Fig. 3) showed that membrane-bound annexin I was ~40 fold more sensitive to trypsin than the free protein.

The increase in reactivity at Lys-9, as shown in the gels of Fig. 1, prompted us to study the reactivity of the peptidic bond 12 to cathepsin D. We found that annexin I bound to PE:PS (3:1) liposomes was proteolyzed much faster (compare the upper with the lower gel in Fig. 4). The optimal pH for cathepsin is 3.4–4.5, but, even when the experiments were run at pH 5.0, the increase in the rate of proteolysis cannot be explained by a lower pH near the negatively charged liposomes: The relatively low surface potential in the PE:PS liposomes and the high ionic strength of the buffer used ensured that the surface pH was not significantly lower than that of the bulk solution (McLaughlin, 1977). Results similar to those presented were obtained when pure PS liposomes were used (not shown).

To check whether the conformational change extends to other residues of the concave face, we studied the reactivity

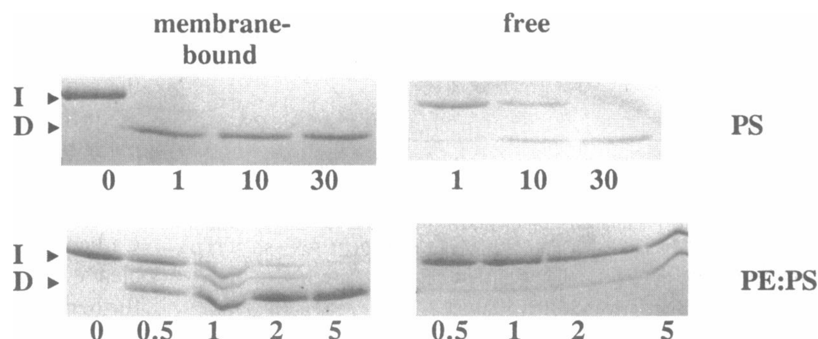


FIGURE 1 Trypsinolysis of membrane-bound and free annexin I. (*Upper gel*) Annexin I bound to PS liposomes: *I*, intact annexin I; *D*, [des 1–26] annexin I. Intact annexin I (66 μg/ml) was bound (*lanes 1–4*) to 0.38 mM PS for 1 min in 0.1 M NaCl, 10 mM Hepes, pH 7.4, 50 μM $CaCl_2$ (nominal). Trypsin (8.5 μg/ml) was added, and at the specified times aliquots containing 4.6 μg annexin I were added to tubes containing trypsin inhibitor. PS was omitted in the experiments with the free protein (*lanes 5–7*). (*Lower gel*) Annexin I bound to PE:PS (3:1) liposomes: (*lanes 1–5*) membrane-bound annexin I, (*lanes 6–9*) free annexin I.

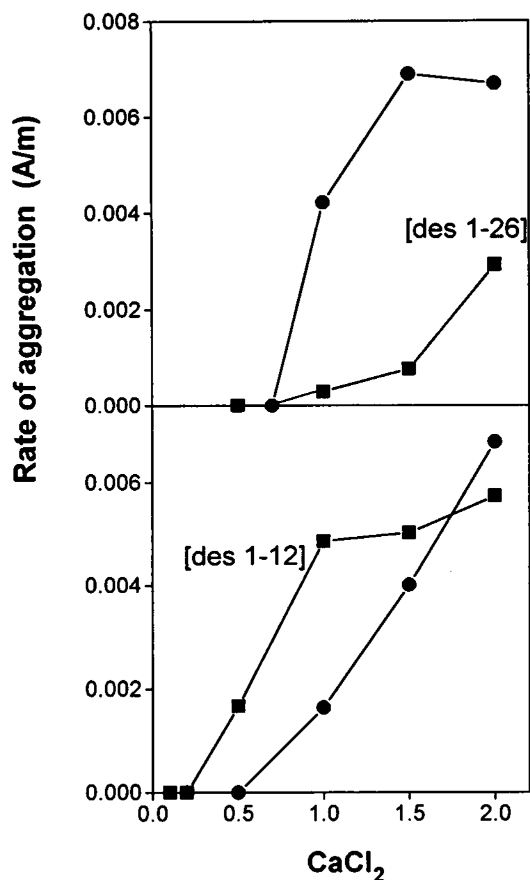


FIGURE 2 Dependence of rate of aggregation of PS liposomes by [des 1–26] and [des 1–12] annexin I on $CaCl_2$ concentration. (*Upper panel*) Aggregation activity of [des 1–26] annexin I. [Des 1–26] annexin I (■) was produced by trypsinolysis as described in Fig. 1. (●) Intact annexin I. Aggregation was started by addition of the specified amounts of $CaCl_2$. PS was 16 $\mu$M, and protein concentrations were 1.4 $\mu$g/ml. (*Lower panel*) Aggregation activity of [des 1–12] annexin I. [Des 1–12] annexin I (■) was obtained as described in the caption to Fig. 4, and the activities were measured as above. (●) Intact annexin I. Rate units are in absorbancy units per minute.

of the unique disulfide bond of annexin I. Reduction of this bond with DTT inhibits the rate of liposome aggregation (Liu and Zimmerman, 1995). As Fig. 5 shows, membrane-bound annexin I (○) reacted much faster with DTT than did the free protein, indicating an increased exposure of the disulfide bond and suggesting that the structural rearrangement includes groups of the annexin core besides the *N* terminus. Calcium at 1 mM (in the absence of PS) had no effect on the rate of reduction (not shown).

Additionally, we used a monoclonal antibody that, according to the manufacturer, binds to the *C*-terminal sequence and therefore can be used to probe groups in the periphery of the concave domain. In agreement with the location of the epitope in the crystalline structure, this antibody does not block annexin I binding to membranes (Meers et al., 1992), but it does inhibit aggregation activity of the intact (Meers et al., 1992), [des 1–26] annexin I (not shown), and membrane-bound annexin I (not shown). When

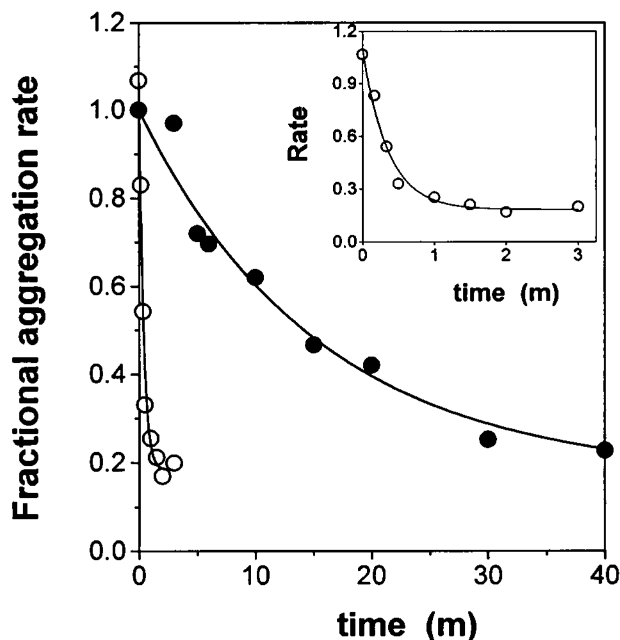


FIGURE 3 Trypsinization of free and membrane-bound annexin I as estimated from aggregation rates measured at suboptimal calcium concentration. Annexin I was incubated with trypsin after binding to PS (○) as described for Fig. 1. Aliquots were removed at the specified times and added to tubes containing trypsin inhibitor. Aggregation was started by addition of 1 mM $CaCl_2$. Normalized rates (calculated as described in Materials and Methods) were then plotted. In the control experiments (●) annexin I was incubated in the absence of trypsin, and aliquots were removed and added to tubes containing trypsin inhibitor for activity determination at the specified times. (*Inset*) Data from membrane-bound annexin I are presented on an expanded time scale. The curves shown are the best fit by the following equation: $y = A1\ \exp(-x/\tau) + A2(1 - \exp(-x/\tau))$, where $x$ is the time in minutes. For the membrane-bound annexin I, $A1 = 1.09$, $A2 = 0.18$, and $\tau = 0.35\ \text{min}^{-1}$. For free annexin I, $A1 = 1.00$, $A2 = 0.142$, and $\tau = 15.84\ \text{min}^{-1}$.

membrane-bound and free annexin I were titrated with the antibody (as measured from inhibition of aggregating activity), the resulting curves were superimposable (not shown). Therefore, the exposure of groups at the *C* terminus in the periphery of the concave surface was not changed by membrane binding.

## DISCUSSION

Induction of a conformational change on $Ca^{2+}$-dependent membrane binding has been shown by fluorimetric and crystallographic studies in the case of annexin V (Concha et al., 1993; Meers and Mealy, 1993). The results presented here demonstrate that binding of annexin I to liposomes increases the reactivity of certain bonds (peptide and disulfide) in the concave face of annexin I to proteolytic or chemical cleavage. This is a strong indication of a major conformational change in the concave face of the molecule. The results thus support our proposal that a second binding site involved in liposomal aggregation is exposed after binding to PS membranes (de la Fuente and Parra, 1995),

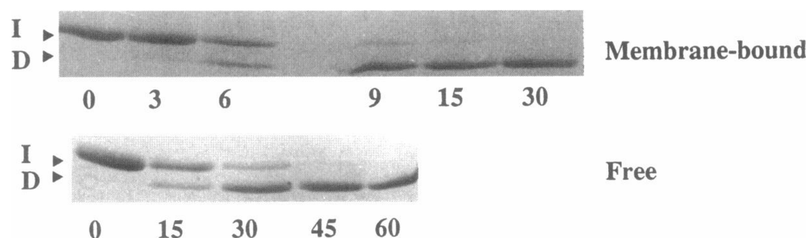


FIGURE 4 Proteolysis of free and membrane-bound annexin I with cathepsin D. Annexin I (64.4 $\mu$g/ml) was incubated with cathepsin D (7.3 $\mu$g/ml) in 0.1 M NaCl, 20 mM Tris acetate, pH 5.0, either bound to 0.38 mM PE:PS (3:1) liposomes (*upper gel*) or free (*lower gel*). Aliquots containing 4 $\mu$g annexin I were taken at the specified times (in minutes) and blocked with pepstatin for analysis. *I*, intact annexin I; *D*, [des 1–12] annexin I.

which requires a membrane-binding-induced conformational change. They also suggest that the concave face contributes to the formation of the secondary site. In this respect, it is worth noting that a number of studies have revealed that the *N* terminus is involved in the aggregation reaction but not in the $Ca^{2+}$-dependent primary binding to the membrane. Thus, proteolysis, phosphorylation, and mutations at the *N* terminus have all been shown to affect dramatically the rate of aggregation (Wang and Creutz, 1992, 1994; Johnstone et al., 1993), whereas an antibody against a part of the *N* terminus inhibits aggregation (Ernst et al., 1991). Additionally, the *N* terminal sequence of annexin I confers aggregating activity on annexin V in a chimera made from the *N* terminal of annexin I and the core of annexin V (Andree et al., 1993; Hoekstra et al., 1993).

Two proposals have been advanced to explain the possible formation of the secondary site, based on the assumption that the secondary site is topologically opposite the primary site. One theory is that the site forms as the result of a reorganization of groups in the concave face, including the *N* terminus. The other is that the annexin molecule opens its two modules so that half of the $Ca^{2+}$ sites would bind the secondary membrane (Meers et al., 1992; de la Fuente and Parra, 1995). In this case, a significant rearrangement in the concave face would also have to occur to accommodate the proposed shift of the lobules. Although at this time neither proposal can be rejected, the second is less probable because of the exceedingly large structural change involved. The results described here, as well as the profound effects caused by changes in the *N* terminus on the rate of aggregation (Wang and Creutz, 1992, 1994; Johnstone et al., 1993), clearly support the first hypothesis. In this case, other groups exposed in the concave face (perhaps the loop connecting repeats 2 and 3 or the *C* helices of some repeats) might collaborate in the formation of the secondary site.

It is noteworthy that, just as primary membrane binding affects the structure of the concave face as described here, phosphorylation, or proteolysis of the *N* terminus, changes the affinity of the convex face for $Ca^{2+}$ (Schlaepfer and Haigler, 1987; Ando et al., 1989). This suggests a structural coupling between the convex and the concave faces of the protein, mediated by the conserved core, so that specific interactions at any one face would induce specific changes at the other. This coupling might play an important role in the physiological interactions of annexin I.

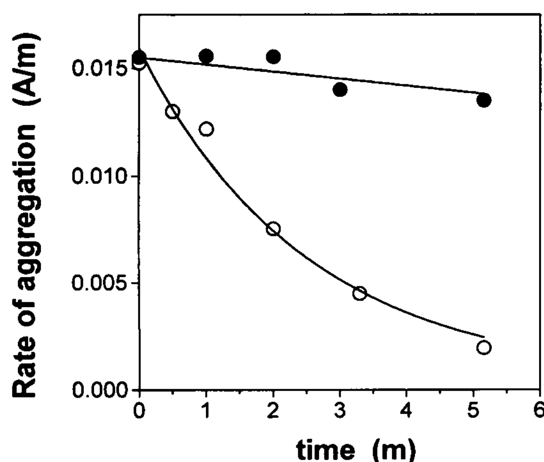


FIGURE 5 Effect of membrane binding on the rate of reduction with dithiothreitol. Annexin I (1.5 $\mu$g/ml) was bound to 10 $\mu$M PS (○). Dithiothreitol (5 mM) was added, and aggregation was started at the specified times with 1 mM $CaCl_2$. For the experiments with free annexin I (●), DTT was incubated with annexin I, and PS and 1 mM $CaCl_2$ were added at the specified times to start aggregation. Curves were drawn by eye.

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