Annexin III, a putative inositol (1,2)-phosphohydrolase, was co-crystallized with inositol 2-phosphate, the inhibitor of the reaction, and its structure was solved to 1.95 Å resolution. No enzyme active site was observed in the structure. Assays for enzymatic activity were also negative. Search for annexin III-inositol phosphate interactions using the BIACore™ system revealed an affinity for inositol cyclic (1,2)-phosphate, suggesting annexin III may sequester the molecule in the cell. The BIACore™ system used with different phospholipids showed that annexin III displays specificity for phosphatidylethanolamine, but not for phosphatidylinositol. Interestingly, a molecule of ethanolamine was found bound to the protein in the crystal structure. Coupled with the fact that this is a particularly abundant phospholipid in granules specific to neutrophils, cells where annexin III is highly expressed, our finding could be pointing to a physiological role of annexin III.

Annexins form a family of proteins that bind phospholipids in a calcium-dependent manner (1, 2). They are widely distributed in different species, tissues, and cell types, with about 18 members having been identified and characterized so far. These proteins are formed by a 4- or 8-fold repeat of 70-amino acid domains that are highly conserved and by a more variable N-terminal segment. It is generally assumed that this variable N-terminal segment is considered to be the locus of calcium channel activity that is considered to be the locus of calcium channel activity observed in vitro for some annexins (1, 11). The conformation of the conserved core of annexin III is fairly close to that of annexin I, with the calcium binding loop in domain III exposed to the solvent. There are three calcium ions bound in this region, a highly occupied type II (12) site and two type III sites with a lower occupancy. Unlike most annexin structures solved so far, the N terminus is well defined in our structure. It lies close to the I + IV module, with the Trp-5 side chain inserted into the hydrophilic central channel.

Nothing in the three-dimensional structure indicated why annexin III among all annexins should have an enzymatic activity. We therefore prepared co-crystals of annexin III with the putative inhibitor (inositol 2-phosphate). In parallel we repeated the experiments that lead to the claim of enzymatic activity (13), which gave negative results. We therefore performed further tests of affinity of annexin III for various inositol phosphates, phosphatidylinositol, as well as other phospholipids, to see if there was any specificity of annexin III toward these compounds, which could explain the results of Ross et al. (14).

MATERIALS AND METHODS

X-ray Crystallography—Annexin III was prepared as before (10). Crystals were obtained by vapor diffusion using ammonium sulfate as a precipitant. The drop of 2 µl contained 18 mg/ml protein, 10 mM CaCl₂, 1 mM MnCl₂, 1 mM inositol 2-phosphate (Sigma) and 25% ammonium sulfate in 50 mM Tris-HCl buffer at pH 7.5. The well contained 50% ammonium sulfate in the same buffer. The crystals are monoclinic, space group P2₁, with cell dimensions a = 42.3 Å, b = 68.7 Å, c = 50.6 Å, β = 94.3°, and one molecule per asymmetric unit. The unit cell volume of these crystals being slightly smaller than the native annexin III, the data were not isomorphous. Data were measured on the D41 station on the DCI synchrotron ring at LURE, Orsay, France which is equipped with a MaResearch imaging plate detector, using a wavelength of 1.38 Å and a crystal-to-detector distance of 103 mm. The data reduction was performed using MOSFLM.
and the CCP4 program suite (15).

The structure was solved by molecular replacement using the structure of native annexin III (10) as a search model, with the program AMoRe (16). The solution was straightforward, since the packing in the crystal is very close to the native crystals.

Refinement was initiated with XPLOR (17), using the rigid-body option with each domain divided into five helices and five loops, followed by conjugate gradient minimization. Further rounds of refinement consisting of simulated annealing were finally completed with PROLSQ (18) to obtain good geometry.

**Enzymatic Activity Assays**—The enzymatic activity was assayed using the protocol described by Ross et al. (13, 19). The activity of recombinant annexin III was compared with that of human placental annexin III prepared according to (20). We used a crude extract from fresh pig lungs as control. 75 mM H-labeled inositol cyclic (1,2)-phosphate (prepared as in Ref. 13) was incubated with 100 μg of either recombinant or placental annexin III, or of the crude cellular extract, for 10 or 20 min at 37 °C, in 50 mM Tris-HCl buffer, pH 7.8, and in the presence of 5 mM MgCl₂. The reaction products were separated on a Dowex formate column with an ammonium formate gradient. Reaction products were evaluated by measuring the radioactivity of the beads. We used GST as a negative control, while a pleckstrin homology domain GST-fusion protein was used as a positive control.

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sensor chip was regenerated after each cycle of measurement by injecting 5 μl of 5 mM EDTA.

RESULTS

X-ray Crystallography—X-ray crystallography data statistics and the refinement results are resumed in Tables I and II. The refinement was started with no solvent molecules defined, and they were progressively added into difference density peaks. All large difference density peaks that could correspond to an inositol phosphate molecule were examined. Only one peak was large enough to attempt the modeling of an inositol phosphate, though it did not refine well. This density lies close to one of the type III calcium sites in domain III, near the surface of the molecule. Since we were refining the structure of native annexin III (at the same time), as well as a mutant annexin III structure (isomorphous to native annexin III), we were surprised to find a very similar density in all these structures, when comparing the refinements. Although fitting this density with water molecules was unsatisfactory, it clearly corresponded to a relatively small molecule. Furthermore, inositol 2-phosphate was present in only one of the three crystallization protocols, while the density had a very similar shape in all. Upon examination of the purification protocol, we decided to model the density as glycerol or ethanolamine. In the end, only ethanolamine could be fitted and refined satisfactorily.

Assays for Enzymatic Activity—While the enzyme is per-
effectively active in the lung extract, producing significant quantities of inositol 1-phosphate, we observe no measurable activity for either placental or recombinant annexin III (Fig. 1). Interestingly, the spontaneous dephosphorylation of inositol cyclic (1–2)-phosphate to inositol is significantly slowed in the presence of annexin III.

Annexin Affinity for Inositol Phosphates and Phospholipids—The quantity of inositol phosphates bound to the different GST-fusion proteins is expressed in percentages, taking the pleckstrin homology domain protein as a reference, in Table III. While annexin V does not bind either of the inositol phosphates tested, annexin III binds inositol cyclic (1–2)-phosphate quite significantly. This measurement correlates well with the observation above, that annexin III protects inositol cyclic (1–2)-phosphate from dephosphorylation.

Figures 2 and 3 summarize the results obtained by flowing of different phospholipids over annexins and the inverse procedure, respectively. As far as the binding of phospholipids to annexins is concerned, our results generally confirm earlier results obtained by classical methods (24). While annexin II...
Annexin III

shows by far the highest binding affinity for all phospholipids tested except PC, annexin III has a significantly higher affinity for PE than either annexin I or V. On the other hand, it is clearly not specific for inositol phosphate head groups. These results are not significantly different when PI or PIP₂ are mixed with PS (not shown).

DISCUSSION

In our hands, the enzyme activity assay described by Ross and Majerus (13) shows quite clearly that annexin III, be it recombinant or prepared from human placenta, has no phosphohydrolase activity. Our purification protocol, starting from placental tissue, differs from that described in Ref. 13 and might be less prone to the co-purification of the true enzyme with annexin III. A recent paper (25) showed equally clearly that the results given in Refs. 9 and 13 were due to the use of inappropriate equipment for the final purification.

In a later paper, the same authors (14) used the transfection of cells by annexin III cDNA to demonstrate that the phosphohydrolase activity was dependent on the expression of annexin III. We can suggest two possibilities for the role of annexin III: either it interacts with the real enzyme and enhances its activity, or it is capable of binding and presenting the substrate. The latter hypothesis is interesting in view of the fact that we found a non-negligible affinity of annexin III for inositol cyclic (1,2)-phosphate, but none for annexin V. Further experiments are necessary to answer this question.

We have not been able to locate a single molecule of the inhibitor in difference electron density maps for crystals of annexin III prepared in the presence of inositol 2-phosphate. It is clear, however, that it had an effect on the crystal formation, since its presence has caused a 2% decrease in unit cell volume, sufficient to render the data non-isomorphous.

The inclusion of inositol 2-phosphate in the crystallization medium has an effect on the conformation of the third domain, which further contributes to non-isomorphism of the crystals. As we noted in our previous paper (10), domain III of annexin III is rotated with respect to domain II, relative to annexins I and V, reducing the symmetry between the two modules of the molecule. Under the influence of inositol 2-phosphate in the crystallization medium, the movement is further accentuated, confirming the notion of domain flexibility in annexins (Fig. 4).

We have modeled the difference density peak found close to a secondary calcium site in domain III as a molecule of etha-nonolamine. As mentioned under “Results,” the purification protocol for annexin III makes use of an ethanolamine buffer. Even though this buffer is not used for crystallization, where we use Tris-HCl buffers, ethanolamine is the only molecule that fits well into the density. This finding would suggest an affinity of annexin III for ethanolamine, which is one of the head groups that occur in natural phospholipids.

The binding of ethanolamine occurs in domain III, which in annexin III has a unique feature: the calcium binding loop, which contains a Trp, in common with annexins IV, V, and VI, where it is buried, however, is exposed to the solvent even in the absence of calcium (10). If we assume that the Trp side chain is important for the anchoring of annexins on the membrane surface, annexin III would always be in a conformation appropriate for binding.

Given the presence of the ethanolamine molecule in our structure, and making use of the sulfate coordinates in an annexin V structure described by Sopkova et al. (26) to fix the position of a phosphatidylserine, we can propose a model for the interaction of the calcium loop of domain III in annexin III with phospholipid headgroups of a membrane (Fig. 5). Our model differs somewhat from the one presented by Swairjo et al. (27), who found the negatively charged glycerophosphoserine, but not the neutral glycerophosphoethanolamine, spanning two calcium ions. We think that modeling one phospholipid per calcium ion is a more realistic representation of the interaction of an annexin molecule with the membrane surface.

Another feature that is unique to annexin III is related to its sequence in this domain. Within a radius of 10 Å we find four negatively charged residues, all on the surface in the proximity of the calcium binding loop. Among these, only Asp-193 (annexin III numbering) corresponds to a residue conserved throughout the annexin family. The others, Asp-151, Glu-194, and Asp-195 occur either in annexins I and II (Asp-151) or in annexin V (Glu-194, Asp-195). The presence of Glu-194 is essential for the formation of the type III calcium site called AB’ in Ref. 27 and Ca₄ in Ref. 28. Glu-194 is also present in annexin IV, where there is no calcium observed in domain III (Protein Data Bank entry 1ANN) and in annexin VI (coordinates not available yet) (29). A comparison of surface charge around the region of the calcium loop in domain III shows a higher negative charge for annexin III, compared with annexins V and I, the only structures with a calcium loop in domain III whose coordinates are available (Fig. 6). One can therefore speculate that this negative charge in annexin III is less favorable to the binding of PS, which is negatively charged, compared with the uncharged PE headgroup.

Thus the results concerning lipid specificity of annexin III are particularly interesting: annexin III does not bind either PI or PIP₂, while it does bind PE. With respect to phospholipid binding, therefore, annexin III does not seem to be relevant as far as inositol headgroups are concerned. If it has a role in inositol phosphate metabolism, it can only be via its relationship with the cellular inositol cyclic (1,2)-phosphohydrolase. On the other hand, annexin III does show specificity for PE (Ref. 24 and our results). This specificity, as well as the location of an ethanolamine molecule near the calcium site of the third domain, could explain the preferential association of annexin III with specific granules of neutrophils, in which this annexin represents about 1% of total proteins. Plasma membrane contains little PE compared with the specific granule membrane (30, 31). It would therefore be very interesting to pursue a thorough analysis of the membrane composition of the different compartments in neutrophils to see whether annexin III association with specific granules is due to the presence of a specific phospholipid, such as PE. This could provide a better understanding of the specific biological role of annexin III in neutrophils.

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