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2D crystal forms of annexin IV on lipid monolayers

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Two-dimensional crystalline arrays of annexin IV were generated by interaction of the purified protein with a phospholipid monolayer. Image analysis of electron micrographs of the protein crystals, which diffracted to 3.5 nm respectively, revealed p6 and p3 symmetry. Annexin IV gave two crystal forms with unit cells of 18 × 18 nm and 28 × 28 nm. The former unit cell was similar to a previously described form of annexin VI. The implications of these observations are discussed.

Annexin; Two-dimensional crystal; Lipid monolayer

1. INTRODUCTION

The annexin family comprises at least 7 different mammalian proteins, all of which bind to cellular membranes in a Ca^{2+}-dependent manner. The annexins are widely distributed, occurring in plants, insects and animals, which suggests that they have important biological roles common to many organisms [1].

All members share a common conserved 70 amino acid repeating unit and the family can be further divided into four repeat and 8 repeat-containing proteins (for a review see [1]). A large volume of biochemical evidence relating to different members of the family has led to suggestions that the annexins mediate phospholipase A_2 inhibition and inflammatory regulation [2], membrane/membrane fusion [3], cytoskeletal organisation [4], blood coagulation [5], signal transduction [1] and/or inositol hydrolase activity [6].

Annexin VI (p68) and annexin IV (p32) are intracellular family members which were originally isolated from T-lymphocytes [7] and bovine tissues, respectively [8], and more recently from human placenta [9]. The primary structure of annexin VI revealed a highly conserved sequence which was repeated eight times and which contained a consensus segment of 17 amino acids that also occurs in the four repeat conserved sequences of annexin IV.

The crystallisation of annexin VI on lipid monolayers has been shown before [10]. The crystals possessed p3 symmetry with a unit cell of about 17.8 × 17.8 nm and resolution of their structure to 5 nm revealed a two-domain protein structure giving a cylindrical molecule of about 10 × 3.5 nm diameter packed as trimers.

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In this study we report the visualisation by electron microscopy (e.m.) of 2D crystals formed by the association of annexin IV with a dimyristoylphosphatidylethanolamine (DMPE) lipid monolayer. The structure of the crystalline areas was enhanced by using computer-assisted image analysis as described by [10]. As well, the structure of the annexin VI 2D crystals were re-analysed using a real space lattice averaging technique, essentially as described by [11], in order to provide more detailed information about the nature of the annexin VI/lipid interaction. The molecular packings of annexins IV and VI are compared and the structure/function aspects of the findings are discussed.

2. MATERIALS AND METHODS

Annexins IV and VI were purified from human placenta as described [9,10], and final protein concentrations determined by the Bradford protein assay using bovine serum albumin as a standard. The purified proteins appeared as a separate band in the case of annexin IV and as a doublet in the case of annexin VI, on SDS-PAGE when stained with Coomassie blue.

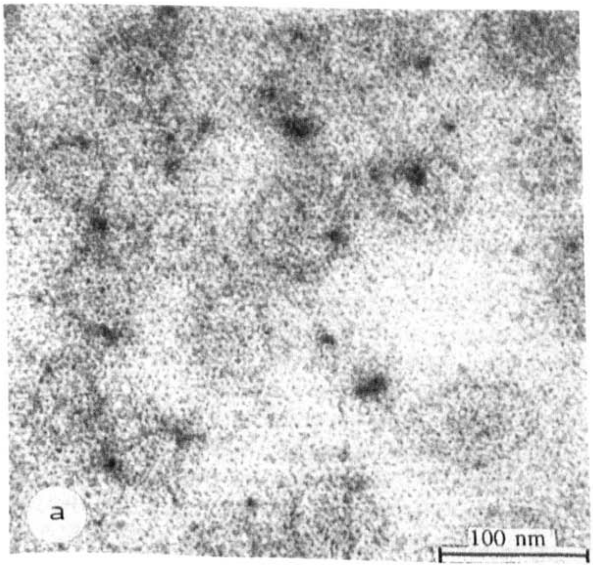
Purified phospholipids were obtained from Avanti (Alabama, USA) and dissolved in chloroform at a concentration of 25 μg/ml. Lipid monolayers were formed on 20 μl drops of 10 mM sodium phosphate buffer (pH 7.4) containing 20 mM NaCl and 1.0 μM Ca^{2+}, as described [10]. Alternatively, lipid monolayers were formed by spreading 1 μl of the lipid solution on clean buffer (as above) contained in a teflon Langmuir trough. This was followed by compression of the lipid monolayer to a surface pressure of 15 mN/m (as described [11]). Protein was added to the aqueous phase (0.15 μg/ml of annexin VI or 0.3 μg/ml of annexin IV), followed by incubation at 37°C for 4 h. Freshly prepared carbon-coated formvar 400 mesh electron microscope grids were used to pick up the lipid monolayer with bound protein, which were examined using a Philips 401 transmission electron microscope.

Computing and image analysis were carried out as described [10] and selected crystalline areas of annexin VI were combined using the real space lattice averaging technique of Saxton and Baumeister [11].

3. RESULTS AND DISCUSSION

Fig. 1A shows an electron micrograph of DMPE/annexin IV monolayers prepared by incubating 0.15 μg of annexin IV/ml under the monolayer at a surface pressure of 15 mN/m for 3–4 h at 37°C. Two-dimensional crystalline domains of the order of 0.5 μm^2 were observed when micrographs were examined using an optical diffractometer (Fig. 2A). Computerised image analyses of selected crystalline areas showed that the crystals diffracted to about 3.5 nm; the unit cell constants of this lattice were $a = b = 17.6$ nm, $\gamma = 60°$. Inspection of the noise-filtered image indicated hexagonal packing. Further averaging of the filtered image based on phase residual analysis was carried out assuming p6 symmetry (Fig. 3A). The distribution of stain excluding density indicated that, under these conditions, the protein monomer has dimensions of about 4.5 × 3.5 nm. Repeat rings of protein (white) form a regular hexamer arrangement containing six protein domains, as shown in the inset in Fig. 3A.

Examination of the diffraction pattern from another region of the electron micrograph (Fig. 1B), of DMPE/annexin IV monolayers, showed a different intensity and spacing, which gave a larger unit cell (Fig. 2B). Computer image analyses of these crystalline



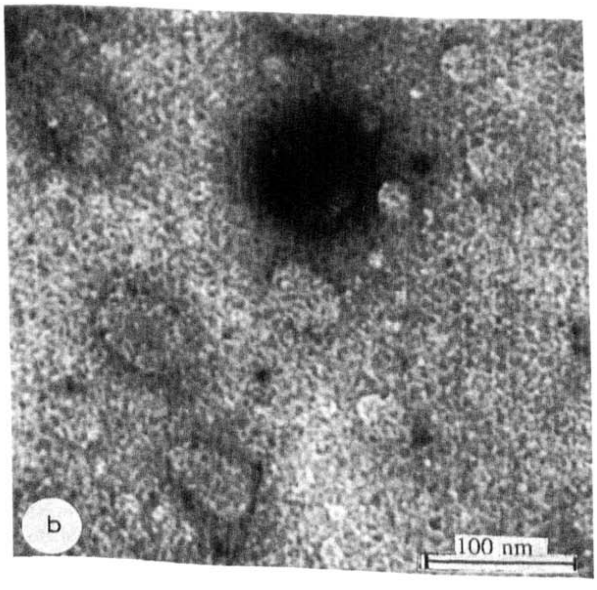
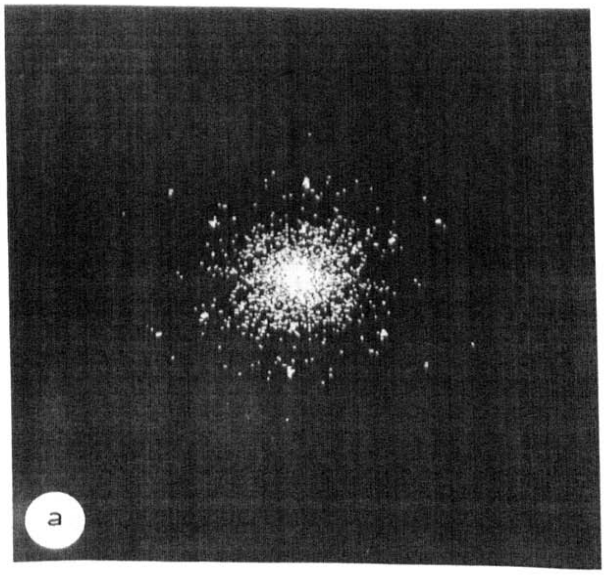


Fig. 1. Electron microscope images, negatively stained with uranyl acetate, of two forms, (A) and (B), of ordered two-dimensional arrays of annexin IV, formed on monolayers of DMPE.



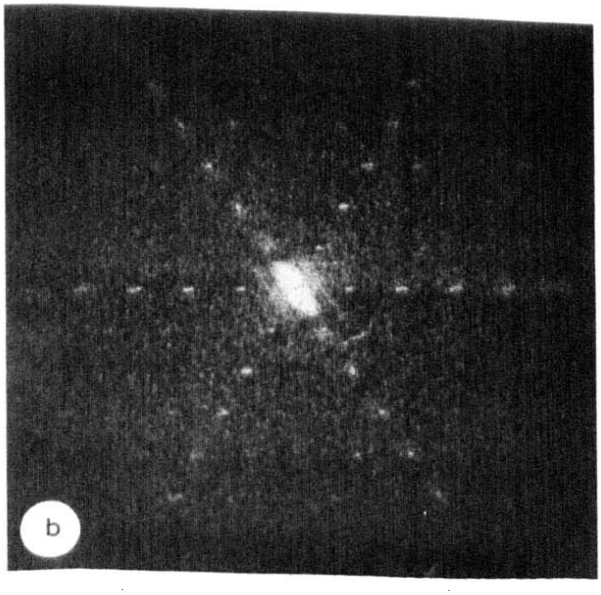


Fig. 2. Computer-calculated diffraction patterns (A and B) corresponding to the crystalline areas of the images shown in Fig. 1A and B, respectively. Diffraction spots extend to the fourth order (3.5 nm).

areas showed that they were in the form of a hexagonal lattice that diffracted to about 3.5 nm; the unit cell constants of this lattice were $a,b = 27.8$ nm, $\gamma = 60°$. Analyses of the phases and inspection of the noise-filtered image (Fig. 3B) again indicated hexagonal packing with p6 symmetry. Repeat rings of protein (white) form a regular hexamer arrangement containing 12 protein domains (5 nm long × 3.5 nm diameter), as shown in the inset in Fig. 3B.

The interaction of annexin IV with PE to form two-dimensional crystals, suitable for analysis by electron microscopy, enables us to compare these crystals with those of annexin VI. It also provides further information about the number of molecules able to pack under a lipid monolayer.

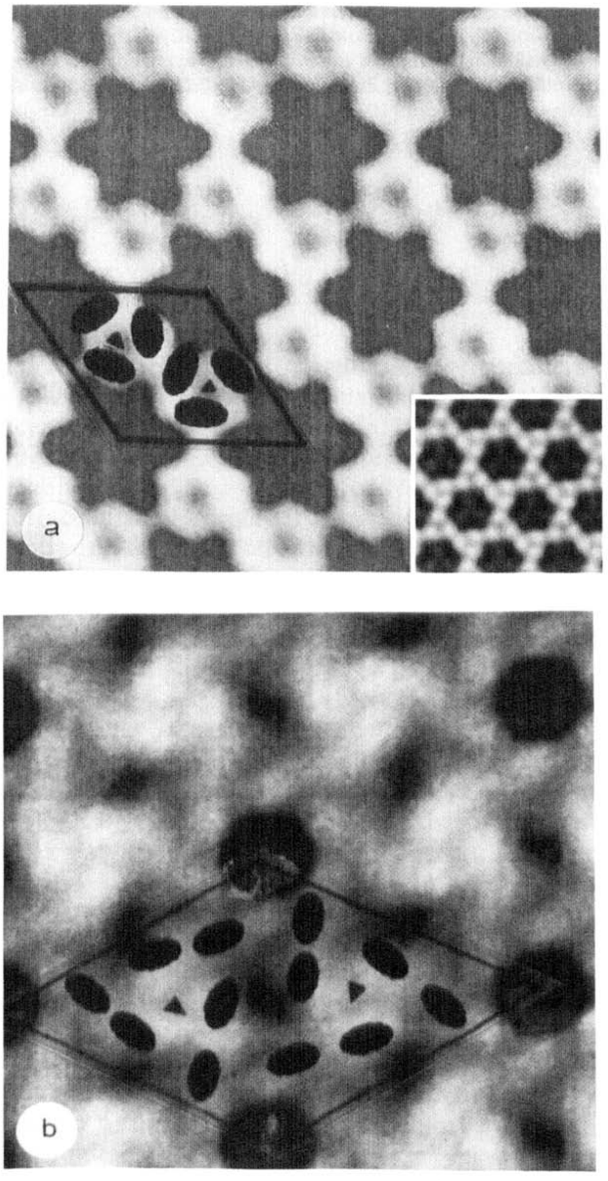


Fig. 3. Noise-filtered images (A and B) after applying p6 symmetry to the crystalline areas of the images shown in Fig. 1A and B, respectively. Rings of protein (white) surround darker stained areas. The annexin IV unit cells are visualised as insets on (A) and (B). The noise-filtered, p3 symmetrised, image of annexin VI, shown as an inset bottom right on (A) has been added for comparison.

Table I

Crystallisation data determined for 2D crystals of annexins IV and VI on lipid monolayers

Protein	Plane group	Unit cell		Molecules/ unit cell
		a (nm)	b (nm)	
Annexin IV (i)	p6	18	18	6
Annexin IV (ii)	p6	28	28	12
Annexin VI	p3	17.8	17.8	6

The plane group symbols are written in Holser notation [13].

Real space averaging of selected areas from electron micrographs of similarly prepared DMPE/annexin VI crystals [10] revealed in greater detail the trimeric arrangement of the six protein domains (inset Fig. 3A). The protein molecules assemble on the monolayer as a trimer of dimers. A comparison of the annexin IV and VI 2D-crystals is shown in Table I.

The crystals we analysed showed either p3 (annexin IV) or p6 (annexin VI) symmetry with different unit cells. The unit cell from annexin VI was of the order of 18 nm^2, containing 3 protein monomers, whilst the two unit cells from annexin IV were respectively 18 nm^2 and 28 nm^2, containing 6 and 12 protein monomers, respectively. For the eight repeat annexin VI protein the size of the protein domains (5 nm long × 3.5 nm diameter), where each domain corresponds to one four repeat unit, is not significantly different from the four repeat annexin IV molecule (5.5 nm long × 3.5 nm diameter). This suggests that the protein/protein contact in dimers of four repeat annexins may be similar to the intra-domain contacts of the eight repeat annexins. The larger unit cell from annexin IV represents a different packing arrangement with protein monomers appearing to form trimers of dimers and giving a denser covering of the lipid monolayer than that seen for annexin VI. The occurrence of a larger unit cell of annexin IV molecules under similar crystallisation conditions indicates that there may not be a particular physiological significance in the way that these molecules interact with the lipid layer.

The existence of two clearly defined domains in the annexin VI monomer as seen in the monolayer crystals compared to the single domain in the annexin IV monomer crystals is interesting in view of the sequence similarity of the two forms. The primary sequence for annexin VI predicts eight homologous repeats whereas the related protein annexin IV has four of these repeats, suggesting that annexin VI is built up of two annexin IV-like units [1]. Of all the annexin repeats those from annexin IV are most like the repeats of annexin VI

(Barton, G., Newman, R.H., Freemont, P.S. and Crumpton, M.J., unpublished observations). The quasi-equivalence of packing of the two units (domains) in the monolayer crystal structure is also consistent with a high degree of similarity between the two units.

We are currently seeking ways of increasing the size of the annexin IV crystals in order to use frozen-hydrated e.m. techniques to extend the resolution beyond the limit imposed by negative stain.

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