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# Crystallization and preliminary X-ray studies of annexin IV (endonexin), a calcium-dependent phospholipid-binding protein

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Annexin IV (endonexin) has been purified from chicken liver and crystallized by the vapour diffusion method. Crystals which diffract to at least 2.2 Å have been obtained. They belong to space group R3 and have unit cell dimensions of $a=b=99.4$ Å, $c=96.2$ Å, $\alpha=90°$, $\beta=90°$, $\gamma=120°$. There is one molecule of 32 500 Da per asymmetric unit.

Annexin; Calcium-dependent phospholipid-binding protein; Crystallization; Endonexin; Chicken liver

## INTRODUCTION

Annexin IV (also referred to as endonexin, protein II or chromobindin 4) is a 32 500 Da member of the annexin family of calcium-dependent phospholipid-binding proteins (for reviews see [1–3]; for nomenclature see [4]). Other well-characterized members of the annexin family include annexin I (lipocortin I), annexin II (calpactin I), annexin V (endonexin II), annexin VI (p68) and annexin VII (synexin). The complete amino acid sequence of annexin IV has been determined [5,6]. In common with other annexins, it consists of a unique N-terminal tail followed by a core of four (or eight in the case of annexin VI) repeats of a highly conserved 70–80 amino acid sequence.

The crystal and molecular structure of annexin V has recently been reported [7,8]. It is folded into four domains, each corresponding to one of the four amino acid sequence repeats. The amino acid sequence of annexin V shows 58% overall identity to annexin IV, with the N-terminal tail regions having a much lower amino acid sequence identity (approximately 28%) than is found in the four repeated sequences of the protein core [9]. The N-terminal region of annexin IV contains a residue phosphorylated by protein kinase C in vitro, whereas annexin V appears not to be a protein kinase C substrate [5,9]. Annexins IV and V have different affinities for phospholipids [10,11] and each has a distinct tissue distribution. Annexin V is found in most cell types, whereas annexin IV has a more restricted distribution, suggesting a more specialized role [12]. It has been proposed that the dissimilar N-terminal tail regions may confer a different function on each annexin [9].

As a means of investigating further the three-dimensional structures of members of the annexin family, we have commenced a crystallographic study of annexin IV. In this paper, we present the first report of its crystallization.

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## 2. EXPERIMENTAL

Annexin IV was purified from chicken liver using a method modified from [13]. Tissue (250 g) was homogenized in a Waring blender with 1.2 l of 0.15 M NaCl/5 mM EGTA/0.25 mM PMSF/10mM Hepes, pH 7.4, and centrifuged in a Sorvall GSA rotor at 20 000 × *g* for 30 min. The supernatant was removed and $CaCl_2$ added to it to a final concentration of 6 mM (1 mM excess). After 15 min on ice, the fraction was centrifuged for 30 min at 20 000 × *g*. The pellet was washed twice with 200 ml of 0.15 M NaCl/1 mM $CaCl_2$/10 mM Hepes, pH 7.4, by resuspension and centrifugation (Sorvall SS-34 rotor, 40 000 × *g* for 30 min), and then twice with 200 ml of 1 mM $CaCl_2$/10 mM Hepes, pH 7.4. The pellet was then resuspended in 75 ml of 10 mM EGTA/10 mM Hepes, pH 7.4, and centrifuged for 30 min at 100 000 × *g*. All steps were performed at 4°C.

The final supernatant was dialysed against 20 mM Hepes, pH 7.4, and further purified using ion-exchange chromatography on DEAE-cellulose equilibrated in the same buffer. The conditions were essentially as described in [13], but using a linear gradient of 0–0.6 M NaCl in 20 mM Hepes, pH 7.4, for elution. Annexin IV is eluted as the major protein peak at approximately 0.08 M NaCl. The peak protein fractions were collected, pooled, dialysed against 20 mM Hepes, pH 7.4, applied again to the DEAE-cellulose column (re-equilibrated with 20 mM Hepes, pH 7.4) and eluted with a linear gradient of 0–0.4 M NaCl in 20 mM Hepes, pH 7.4. This procedure removed a number of minor contaminants. The fractions containing annexin IV were then combined and dialysed extensively at 4°C against 20 mM Tris-HCl, pH 8.0, containing 10 mM $NaN_3$. In order to concentrate the protein sufficiently for crystallization, the protein solution was placed in dialysis tubing, covered in polyethylene glycol 20 000 and left at 4°C for several hours. By this means, small volumes of highly concentrated protein could be obtained, typically 1 ml at a concentration of

10 mg/ml. Protein concentrations were measured using BCA reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [14]. Gels were stained with Coomassie brilliant blue.

The purified annexin IV was used in hanging drop crystallization trails [15]. The best quality crystals were grown at 18°C from 10 μl drops containing 5 μl of protein solution (10 mg/ml protein in 20 mM Tris-HCl, pH 8.0) mixed with 5 μl of precipitant (2.1 M ammonium sulphate in 20 mM Tris-HCl, pH 8.0, with 1 M $CaCl_2$ added to give a final calcium concentration of 10 mM) suspended above reservoirs containing 1 ml of the same precipitant. Crystals first appear after about one week and growth appears to be complete in 2–3 weeks. The final size of crystals is 0.2 × 0.15 × 0.1 mm. Crystals diffract to beyond 2.2 Å and are stable to X-rays (produced by a Rigaku RU-200B rotating anode with a 200 μm focus operating at 45 kV and 60 mA) for at least five days of continuous exposure. A good quality native data set has been collected using a Siemens X-100A area detector mounted on the Rigaku.

## 3. RESULTS AND DISCUSSION

Annexin IV is the major annexin isolated from chicken liver using the method described. Large amounts, approximately 40 mg/kg, can be obtained in a highly purified form. The initial homogenate and final supernatant of the preparation are shown in Fig. 1a and b. On ion-exchange chromatography on DEAE-cellulose, annexin IV elutes as the major protein peak at approximately 0.08 M NaCl. Fig. 1c shows the purified protein, which migrates as a single spot on two-dimensional gel electrophoresis at 32 500 Da, p*I* approximately 5.6 (not shown). For crystallization studies, the purified annexin IV is dialysed and concentrated as described in section 2.

A typical crystal is shown in Fig. 2. A combination of precession photography (Fig. 3) and autoindexing of the area detector data has established that the crystals are trigonal, space group R3, with unit cell parameters: $a = b = 99.4$ Å, $c = 96.2$ Å, $\alpha = 90°$, $\beta = 90°$, $\gamma = 120°$. The volume per unit protein molecular mass is 2.18 $Å^3$/Da for one molecule of molecular weight 32 500 per asymmetric unit ($Z = 9$ for hexagonally indexed R3). This corresponds to a solvent content of approximately 56%, which is well within the range observed for other proteins [16].

The results described here represent the first report of annexin IV crystals to date. Preliminary crystallographic studies of a further member of the annexin family, annexin VI (p68), have also been described [17]. The annexin IV crystals described here have the same group (R3) and almost identical unit cell dimen-

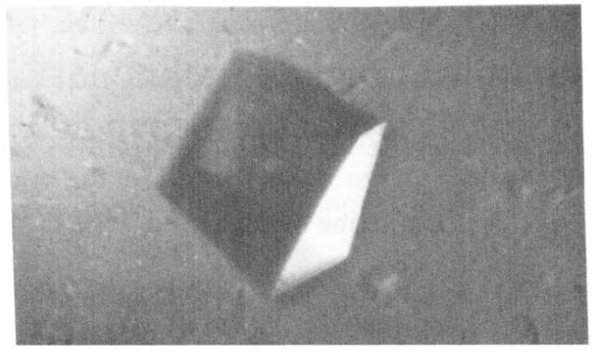

Fig. 2. Crystal of annexin IV from chicken liver. The longest side of the crystal is 0.3 mm in length.

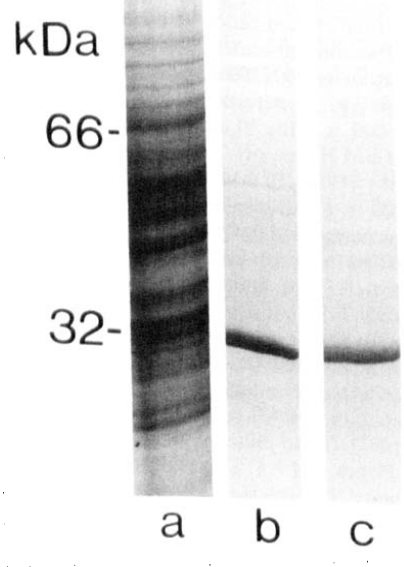


Fig. 1. 10% SDS-polyacrylamide gel electrophoresis of stages in the purification of annexin IV from chicken liver. (a) Homogenate of chicken liver; (b) final supernatant of the liver preparation; (c) purified annexin IV eluted from DEAE-cellulose.

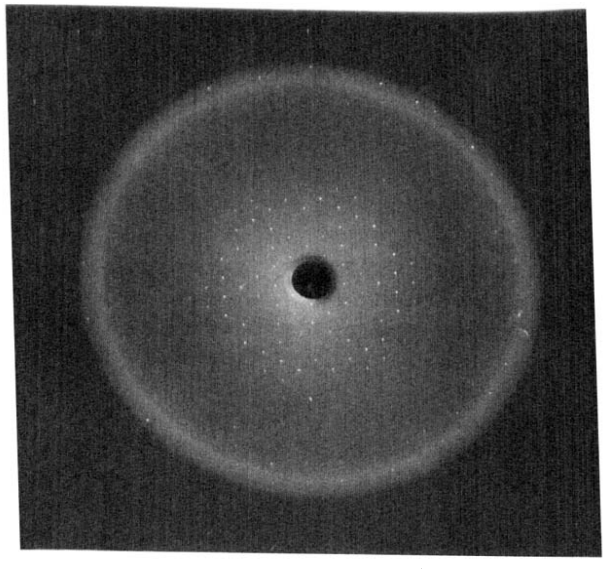

Fig. 3. Precession photograph of annexin IV from chicken liver. This 10° picture (hk0) was taken at a crystal to film distance of 100 mm on a Rigaku rotating anode X-ray generator operating at 45 kV and 60 mA with an exposure time of 15 h. The 10° precession photograph shows strong diffraction to 4.4 Å. Diffraction to beyond 2.2 Å can be obtained.

sions to the annexin V crystals whose structure has been determined (for annexin V, $a = b = 99.6$ Å, $c = 96.4$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$) [8]. Further crystallographic studies of annexin IV are underway to determine its three-dimensional structure.

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