Purification, Crystallization, and Preliminary X-ray Diffraction Analysis of Rat Kidney Annexin V, a Calcium-dependent Phospholipid-binding Protein*

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We have purified annexin V, a monomeric 35-kDa protein, from rat kidney using calcium-dependent phospholipid chromatography. The identity of annexin V was confirmed by immunoblot analysis using monospecific anti-annxin V antibody. Large single crystals of annexin V in the presence of calcium have been grown from ammonium sulfate under a variety of conditions, with an optimum pH range of 7.5-8.0. The crystals diffract to at least 2.2 Å Bragg spacing and are stable to x-rays. Preliminary crystallographic analysis reveals the space group to be R3, with hexagonal cell dimensions of \( a = b = 156.8 \text{ Å} \) and \( c = 36.9 \text{ Å} \), and there is one molecule/asymmetric unit.

Calcium acts as a transient second messenger in response to a wide spectrum of cell-specific stimuli such as growth factors, hormones, and other intercellular transmitters. The calcium signal is responsible for the regulation of many cellular activities including cell growth and division, motility, adhesion, ion transport, and secretion (for review, see Campbell, 1983). Intracellular calcium-binding proteins are the effectors that mediate and coordinate stimulus-response coupling through the calcium system (Kaetzel et al., 1989). The calmodulin/troponin C superfamily is a well characterized group of calcium-binding proteins that coordinate the calcium ion within a helix-loop-helix domain termed an “E-F hand” by Kretsinger (1975). This structural domain is widely found in nature and provides proteins a mechanism to be regulated by changes in free calcium. Crystal structures of several E-F手 calcium-binding proteins have been determined. Members of this family generally express their functions, where known, through calcium-dependent protein-protein interactions.

More recently, several calcium-dependent phospholipid-binding proteins have been characterized (for review, see Crompton et al., 1988). So far, seven distinct members of this novel family, termed annexins I–VII (Geisow, 1986; Kaetzel and Dedman, 1989) have been identified. They are related through high degrees of sequence similarities and appear to be structurally and functionally distinct from the calmodulin/troponin C family. Although these calcium/phospholipid-binding proteins are composed of conserved repeated domains of about 70 residues, the E-F hand structure is not predicted from primary sequence data (Pepinsky et al., 1988; Kaplan et al., 1988; Crompton et al., 1988; Sudhof et al., 1988). Microcrystals of p68 (annexin VI), the only annexin with eight rather than four repeated domains, were reported recently by Newman et al. (1989).

Annexin V, a 35-kDa monomeric protein, has four repeated domains as shown by its deduced amino acid sequence (Pepinsky et al., 1988). As with other annexins, it has been referred to previously by several names, including placental anticoagulant protein or PAP-I (Funakoshi et al., 1987),endonexin II (Haigler et al., 1987), lipocortin V (Pepinsky et al., 1988), and 55γ calcium-binding (Kaetzel et al., 1989).

In order to elucidate the mechanism by which the annexin family of proteins coordinates calcium ions and phospholipids, we have grown large single crystals of annexin V in the presence of calcium which are suitable for high resolution x-ray structure determination. To date, these are the only such annexin crystals that have been reported.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure ammonium sulfate and buffers were obtained from Aldrich and used without further purification. Bovine brain lipid extract and phenyl-Sepharose resin were purchased from Sigma. For crystallization, ultrapure water from a Hydro Services System was used in all solutions.

Purification of Annexin V—The total set of calcimedins, including annexins III–VI, was purified as described previously (Kaetzel et al., 1989) from rat kidney using calcium-dependent phospholipid chromatography. The protein mixture was then applied to a phenyl-Sepharose column (2.5 x 20 cm) in the presence of 200 µM free calcium. Under these conditions, the 30-kDa calcimedin and calmodulin are bound to the column. The unbound protein was applied to a Mono Q column attached to a Dionex high performance liquid chromatography system. The proteins were eluted with a linear 0-300 mM NaCl gradient at a flow rate of 1.0 ml/min; 1-ml fractions were collected and subjected to electrophoresis to confirm purity (Fig. 1). Each fraction was then subjected to immunoblot analysis using monoclonal anti-annexin III, IV, V, and VI antibodies to confirm the identity of annexin V (Kaetzel et al., 1989). Fractions 36, 37, and 38 were confirmed to be solely annexin V. These fractions were pooled, dialyzed exhaustively against Nanopure water (Barnstead), and lyophilized.

Protein solutions used to produce crystals tested negative for the presence of phospholipids, as determined by phosphate assay (Van Veldhoven and Mannsma, 1987). Protein concentrations were determined by quantitative amino acid analysis, using an amino acid
Preliminary X-ray Crystallographic Studies of Annexin V

Crystallization of Annexin V—Large single crystals of annexin V were grown by hanging droplets or microdialysis methods from solutions containing 10-30 mg/ml protein, 40-60% ammonium sulfate, 5-10 mM CaCl₂, 50 mM HEPES or HEPPS buffer, 1 mM dithiothreitol, and sodium azide. Crystals of annexin V from rat liver, kidney, or testis grew under very similar conditions as hexagonal or pyramidal plates, triangular prisms, or irregular rhombohedrons. The best crystals grew at room temperature from solutions buffered at pH 7.5-8.0. Smaller crystals grew outside this range, from pH 6.5 to 8.5. Single crystals appeared within a week, and frequently continued to grow for several more weeks. Protein stability under these crystallizing conditions is excellent: hanging droplets can be pooled after several months' growth time, redissolved in buffer as necessary, and used to produce crystals again.

X-ray photographs were taken with a Supper precession camera fitted to an Elliott GX-6 rotating anode, typically operated at 1-kilowatt power with a 100-μm focusing cup. X-rays of 1.542 Å wavelength are produced from a copper target and nickel filtered.

RESULTS AND DISCUSSION

Of the annexin V crystals grown, we have characterized most fully by x-ray diffraction methods those from rat kidney protein. The largest crystals, which grow to 0.3-0.4 mm on a side, exhibit very good diffracting power. These crystals show many reflections to at least 2.2 Å Bragg spacing on x-ray still photographs. Even those of dimensions 0.2 × 0.1 × 0.05 mm³ diffract surprisingly well considering their small size and can be examined by means of precession photographs. In all crystals, the physical location of the 3-fold symmetry axis is obvious from crystal morphology. With the hexagonal plates, for example, this axis emerges perpendicularly from the face of the plate. Crystal deterioration in the x-ray beam is slow: one crystal was photographed continuously for a week before it showed significant loss of diffraction beyond 3 Å.

The rat kidney annexin V crystals exhibit diffraction symmetry characteristic of the R3 space group. In the hkl zone (Fig. 2), only reflections corresponding to \(-h + k = 3n\) occur. In the h0l (or 0kl) zone, reflections corresponding to \(h + l = 3n\) are present. In the hkl zone, a \(l = 3n\) pattern is observed. All zones except the hkl exhibit 2-fold rotational symmetry. Hexagonal unit cell dimensions are \(a = b = 156.8\) Å, and \(c = 36.9\) Å.

The hexagonal unit cell in the R3 space group has nine equivalent positions. The unit cell volume of the annexin V crystals is 785,700 Å³. With nine copies of the 35,600-dalton protein in the unit cell, a solvent content parameter, \(V_m\), of 2.45 Å³/dalton is calculated, a typical value by the criteria established by Matthews (1968). The asymmetric unit there-
fore corresponds to one annexin molecule.

In summary, we have purified and crystallized rat kidney annexin V in a form suitable for further x-ray crystallographic analysis. We have initiated a search for heavy metal derivatives preliminary to isomorphous replacement, and have observed diffraction changes in crystals of at least one mercury derivative. We anticipate that the quality of these crystals will be suitable for complete crystallographic structure determination of annexin V.

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REFERENCES
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