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Crystallization and preliminary X-ray analysis of full-length annexin I comprising the core and N-terminal domain

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Annexin I, a member of the annexin family of Ca^{2+}- and phospholipid-binding proteins, has been crystallized with the complete N-terminus. Annexins are structurally divided into a conserved protein core and an N-terminal domain that is variable in sequence and length. Three-dimensional structures of annexins comprising the protein core and a short N-terminal domain (annexins III, IV, V, VI, XII) or a truncated form almost completely lacking the N-terminal domain (annexins I and II) have been published so far. Here, the crystallization of annexin I comprising not only the core but also the complete N-terminal domain is reported. The crystals belong to the space group $P2_12_12_1$, with unit-cell parameters $a = 63.6$, $b = 96.3$, $c = 127.4$ Å, and diffract to better than 2 Å. Assuming a molecular weight of 38.7 kDa for annexin I and an average value of 2.5 Å^3 Da^{-1} for V_M, two molecules per asymmetric unit are present.

1. Introduction

The annexins comprise a family of soluble Ca^{2+}- and phospholipid-binding proteins (for reviews, see Gerke & Moss, 1997; Liemann & Huber, 1997; Swairjo & Seaton, 1994; Raynal & Pollard, 1994). Each member of this protein family contains a conserved protein core that includes the calcium- and phospholipid-binding sites, and an N-terminal domain that is variable in sequence and length for each annexin. The N-terminus differs in length between 11 and 19 (annexins III, IV, V, VI, X, XII, XIII), 33 and 42 (annexins II and I) and more than 100 residues (annexins VIII and XI). It harbors binding sites for S100-protein ligands (annexin I/S100A11, annexin II/S100A10, annexin XI/S100A6) and various phosphorylation sites (*e.g.* annexin I, annexin II) for serine/threonine and tyrosine.

Although highly similar in the three-dimensional structure of the protein core, different annexins exhibit diverse biochemical and functional properties. These properties include the regulation of membrane–membrane and membrane–cytoskeleton contacts, membrane organization and ion currents across membranes. To a large extent, the specific properties of each annexin are likely to be mediated by the variable N-terminal region. However, no structure of an annexin with a long N-terminal domain (more than 30 amino acids) has been published so far because the N-terminal domain is easily lost through proteolysis during the process of purification. The annexin with the longest N-terminal domain to be structurally determined by X-ray crystallography is annexin III at 1.78 Å resolution comprising a short N-terminal domain with 16 amino acids (Favier-Perron *et al.*, 1996).

Thus, the structure of a long N-terminal domain and its interaction with the core domain is expected to reveal new insights into the function of annexins. Annexin I has been shown to aggregate model membrane vesicles and chromaffin granules (secretory vesicles from the adrenal medulla) in the presence of Ca^{2+} ions (de la Fuente & Parra, 1995; Wang & Creutz, 1994). The aggregation property of annexin I strongly depends on the N-terminal domain, whereas the membrane binding is mediated *via* the core domain. A chimera comprising the core domain of annexin V and the N-terminal domain of annexin I is capable of membrane aggregation, while annexin V is not able *per se* to induce membrane or vesicle aggregation (Bitto & Cho, 1998; Andree *et al.*, 1993). Modification of the N-terminal domain by phosphorylation of Tyr21 alters the Ca^{2+}-dependent binding and aggregation properties of annexin I with artificial vesicles and chromaffin granules (Schlaepfer & Haigler, 1987; Wang & Creutz, 1992). Another regulatory mechanism of the aggregation property or other membrane-related properties of annexin I *in vivo* could be binding to S100A11, a member of the S100 protein family of calcium-binding proteins. Biochemical experiments have shown that the binding of the S100A11 dimer is mediated by the N-terminal domain of annexin I (Seemann, Weber & Gerke, 1996; Mailliard *et al.*, 1996). In 1993, the three-dimensional structure of an

Table 1
Data-collection statistics.

Crystal data	
Space group	$P2_12_12_1$
Unit-cell dimensions (Å,°)	a = 63.6, b = 96.3, c = 127.4, $\alpha = \beta = \gamma$ = 90
Data collection	
Total No. of reflections	679717
No. of unique reflections	73364
Overall completeness (%)	93.0
Completeness (1.83–1.80 Å) (%)	99.4
$I/\sigma(I)$ (overall)	14.6
$I/\sigma(I)$ (1.83–1.80 Å)	1.6
$R_{\rm merge}$† (%)	8.2
Mosaicity (°)	0.57

† $R_{\rm merge}$ $(I) = \sum_{hkl}\sum_i|I_{hkl,i} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_i|I_{hkl,i}|$, where $\langle I_{hkl}\rangle$ is the average intensity of the multiple $I_{hkl,i}$ observations for symmetry-related reflections.

annexin I derivative lacking the first 32 amino acids (containing only the last nine amino acids of the N-terminal domain) was published (Weng *et al.*, 1993). However, as mentioned above, certain characteristics of annexin I such as vesicle aggregation are mediated only by the complete N-terminal domain. Recently, the structure of the S100A11 dimer in complex with a short N-terminal peptide of annexin I comprising residues 2–12 has been published (Rety *et al.*, 2000; PDB code 1qls). While this structure showed an α-helical conformation for these 11 residues, it did not show the conformation of the other 29 residues of the N-terminal domain of annexin I, nor did it show how the N-terminal domain of annexin I interacts with its core domain.

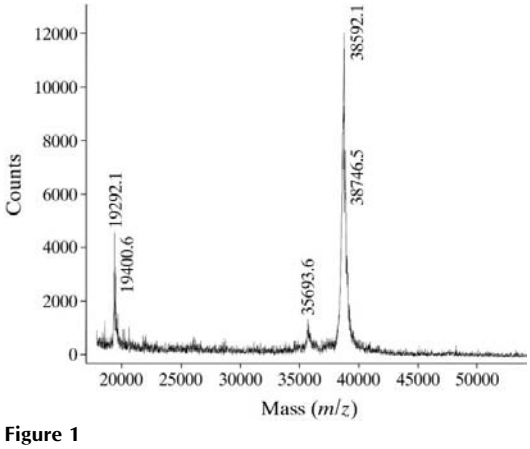

Figure 1
Mass spectra of dissolved annexin I crystals. The MALDI technique was performed to prepare the protein sample using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. The sample was analyzed with the time-of-flight (TOF) mass spectrometer Voyager DE-Pro (Perceptive Biosystems). The observed peaks reflect the following annexin I species: m/z = 38 746.5, annexin I monomer (calculated mass = 38 744 Da); m/z = 38 592.1, annexin monomer minus the first methionine residue; m/z = 19 400.6 and 19 292.1; doubly charged species (m/z = 38 746.5 and 38 592.1, respectively); m/z = 35 693.6, annexin I derivative lacking the first 26 amino acids.

We are interested in the structural requirements and the role of the N-terminus in membrane aggregation or fusion by annexin I. To this end, we have crystallized the full-length recombinant porcine annexin I composed of the core and N-terminal domain in order to determine the high-resolution three-dimensional structure of the intact protein by X-ray crystallography.

2. Materials and methods

2.1. Protein expression, purification and mass spectrometry

The cDNA of porcine annexin I was cloned into an *Eco*RI-linearized pKK223-3 vector and expressed in *Escherichia coli* BL21(DE3)pLysS cells (Seemann, Weber, Osborn *et al.*, 1996). The annexin I monomer contains 346 amino acids, with the N-terminal domain comprising residues 1–41 and the core domain comprising residues 42–346. The primary sequence of porcine annexin I has been published by Seemann, Weber, Osborn *et al.* (1996). Annexin I was purified according to Rosengarth *et al.* (1999). This specific purification protocol ensures, in contrast to the common purification *via* calcium-dependent phospholipid binding, that the N-terminal domain is not proteolytically cleaved. The protein was stored in 50 m*M* MES–NaOH pH 6.4, 150 m*M* NaCl, 1 m*M* EGTA and was used without further changes of the buffer for the crystallization setup.

The integrity of the full-length protein was verified by MALDI–TOF mass spectrometry (shown in Fig. 1). A solution of 10 mg ml^{-1} 3,5-dimethoxy-4-hydroxy-cinnamic acid in 30% acetonitrile/70% water with a TFA content of 0.1% was used as matrix solution. The protein solution was mixed with this matrix solution and was examined with a Voyager DE-Pro mass spectrometer (Perceptive Biosystems).

2.2. Crystallization

Crystals were initially grown by the hanging-drop technique using Hampton Crystal Screens (Hampton Research, Laguna Hills, CA, USA; Jancarik & Kim, 1991). The protein solution was mixed with the respective crystallization solution (3 µl each) at 277 K on a cover slip that was sealed with silicon oil over a well containing 500 µl of the respective crystallization solution. Using a protein stock solution of 28 mg ml^{-1}, crystals were observed after 13 d incubation at 277 K in solution number 4 of Crystal Screen I (2.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5). A fine grid search around these conditions revealed that increasing the ammonium sulfate concentration to 2.2 *M* resulted in crystal growth in 2 d, yielding optimized crystals with average dimensions of 0.2 × 0.2 × 1 mm.

2.3. Data collection and analysis

X-ray diffraction data were collected at beamline 5.0.2 at the Advanced Light Source (ALS), Berkeley, CA, USA. The wavelength of the synchrotron radiation was 1.0 Å and a 2 × 2 array CCD detector from Area Detector Systems Corporation, San Diego, USA was used. The crystals were soaked in mother liquor containing 20% glycerol as a cryo-protectant for a few seconds before being flash-cooled in liquid nitrogen. 135 images with a $\Delta\varphi$ of 1° were collected from one crystal at 100 K. Data analysis was carried out using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Protein expression, purification and crystallization

The purification of annexin I resulted in the full-length protein as confirmed by mass spectrometry. Crystals were grown by the hanging-drop method in 2.2 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5. To date, no crystals of full-length annexin I have been grown under any other Hampton Crystal Screen I or II condition, nor in the presence of calcium ions. The crystals are needle-shaped and exhibit average dimensions of 0.2 × 0.2 × 1 mm. They belong to the space group $P2_12_12_1$, with unit-cell parameters a = 63.6, b = 96.3, c = 127.4 Å.

To verify that the crystals contained full-length annexin I, we also performed mass spectra of dissolved crystals (Fig. 1). The spectra showed two major peaks. One peak at m/z = 38 746.5 agrees with the calculated molecular weight for full-length porcine annexin I (38 744 Da). The second peak at m/z = 38 592.1 corresponds to the mass of one annexin monomer minus the first methionine residue. The spectra also showed a minor peak at m/z = 35 693.6 that reflects the theoretical mass of an annexin I derivative lacking the first 26 amino acids. The intensity of this peak is very low, suggesting

a population of less than 5%. The double peak at m/z = 19 292.1/19 400.6 corresponds to the doubly charged species with a molecular weight of 38 592.1/38 746.5 Da.

3.2. Data collection and analysis

The crystals diffracted to 1.8 Å resolution at beamline 5.0.2 at the Advanced Light Source (ALS), Berkeley, CA, USA. The data set collected shows an overall completeness of 93% and an $R_{\rm merge}$ of 8.2%. Unit-cell parameters and data-reduction statistics are listed in Table 1.

The volume of the unit cell is 780 749 Å^3. This volume divided by four (the z value for space group $P2_12_12_1$) and again by 2.5 Å^3 Da^{-1}, which is the average density or $V_{\rm M}$ for crystals of soluble proteins (Matthews, 1968), yields an approximate molecular weight of 78 kDa per asymmetric unit. This value suggests two annexin I molecules per asymmetric unit.

Further investigation of the diffraction data using a Patterson self-rotation search (program *X-PLOR*; Brünger, 1992) did not reveal any twofold axes other than the expected crystallographic ones along the principal axes.

3.3. Molecular replacement

Patterson cross-rotation and translation searches (molecular replacement, program *X-PLOR*) with the previously reported coordinates of N-terminally truncated annexin I (Weng *et al.*, 1993; PDB code 1ain) yielded two solutions related by a local twofold axis which runs parallel to the crystallographic *y* axis. This finding explains why no local twofolds could be identified with Patterson self-rotation searches. Crystal-packing analysis with both copies of the search molecule placed did not reveal any steric clashes. Moreover, there appears to be enough room for the extra 32 residues per molecule of the N-terminal domain that are expected to be present in the full-length molecule. The initial crystallographic R factor was 47.2% and dropped to 42.7% after 20 cycles of rigid-body refinement (12 parameters). Extensive model building and refinement failed to produce interpretable maps for the previously undetermined part of the N-terminal region (residues 1–32). A search for model-independent experimental phases using the multiple isomorphous replacement (MIR) method (Stout & Jensen, 1968) is in progress.

4. Conclusions

We have obtained crystals of full-length annexin I that diffract to 1.8 Å resolution at the Advanced Light Source, Berkeley, CA, USA. The $V_{\rm M}$ of the unit cell and the preliminary molecular-replacement solution suggest two molecules per asymmetric unit. The phase information from the molecular-replacement dimer was not sufficient to allow fitting of the N-terminus.

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