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Crystallization and preliminary X-ray analysis of human grancalcin, a novel cytosolic Ca^{2+}-binding protein present in leukocytes

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Recombinant human grancalcin, a calcium-binding protein from leukocytes, has been crystallized in the presence or absence of Ca^{2+} by the vapor-diffusion method. Two crystal forms of apo grancalcin were obtained: space group $P2_1$, with unit-cell parameters a = 48.4, b = 81.1, c = 46.6 Å, β = 111.3°, diffracting to 1.9 Å, and space group $C2$, with unit-cell parameters a = 97.0, b = 51.9, c = 75.9 Å, β = 108.5°, diffracting to 2.4 Å. Crystals were also grown in the presence of 5 m*M* Ca^{2+}. They also belong to space group $C2$, with unit-cell parameters a = 97.4, b = 50.3, c = 77.6 Å, β = 108.2°, which are very similar to the second apo grancalcin form. These crystals diffract to 2.5 Å.

1. Introduction

Neutrophilic granulocytes play an important role in the defense mechanisms of mammalian organisms through the efficient killing and digestion of invading microorganisms. There are two main events involved in this function: the generation of $O_2^{\cdot}$ and other oxygen radicals and the release of granule contents into the phagosome (*i.e.* degranulation). The fusion of azurophilic and specific granules with the phagosomal membrane results in the delivery of many proteolytic and saccharolytic enzymes to the phagosomes (Cohn & Benson, 1965; Bainton, 1973). Though the mechanism of signal transmission which leads to activation of these two cellular events is not fully understood, intracellular Ca^{2+} is an important second messenger for these processes (Sengelov *et al.*, 1993). Grancalcin is a cytosolic Ca^{2+}-binding protein which binds reversibly to secretory vesicles and plasma membranes in the presence of physiological concentrations of Ca^{2+} in human neutrophils and might therefore play a role in the regulation of granule–membrane fusion and degranulation (Lollike *et al.*, 1995).

Grancalcin is specifically associated with cells originating in the bone marrow. It is particularly abundant in neutrophils and monocytes and is present in smaller amounts in lymphocytes. The protein consists of 217 amino acids and has a molecular weight of 22.4 kDa. The N-terminus of grancalcin is acetylated *in vivo* (K. Lollike, unpublished data). Grancalcin belongs to a family of calcium-binding proteins characterized by the presence of five EF-hands, together with calpain, sorcin, ALG-2, peflin and yeast YG25 protein (Maki *et al.*, 1997; Kitaura *et al.*, 1999). Presently, the Ca^{2+}-binding domain VI of calpain is the only member of this family for which the three-dimensional structure is known (Blanchard *et al.*, 1997; Lin *et al.*, 1997). In addition to the EF-hand domains, grancalcin contains an ~50 residue-long N-terminal region rich in glycine and hydrophobic residues. Like sorcin and peflin, grancalcin exists in solution as homodimers (Teahan *et al.*, 1992). Members of this family show a surprising diversity of functions: grancalcin participates in granule exocytosis, sorcin binds to and modifies the cardiac ryanodine calcium channel, ALG-2 plays a role in apoptosis and the calcium-binding domains of calpain play a regulatory role in the activation of this cysteine protease.

Based on fluorescence and circular dichroism measurements, a Ca^{2+}-binding-induced conformational change of a grancalcin dimer may take place upon which there is an increase in the hydrophobic character of the protein surface (K. Lollike *et al.*, in preparation). In order to investigate the three-dimensional structure of grancalcin and to determine the character of the proposed conformational rearrangement, we have crystallized this protein in the absence and in the presence of Ca^{2+}. Several different crystal forms have been obtained and diffraction data have been collected.

2. Methods and results

2.1. Protein purification

Purification followed the previously published procedure (Lollike *et al.*, 1995) with some modifications. Recombinant grancalcin (*r*-grancalcin) was expressed in *Escherichia coli* strain XL1-blue (Stratagene, La Jolla, CA, USA) containing the vector pGEX2T (Pharmacia) with the coding region of grancalcin in

frame with the GST gene. This strain was inoculated into 50 ml broth culture (1% NaCl, 1% tryptone and 5% yeast extract, pH 7.2–7.5, Bie and Berntsen, Rødovre, Denmark) and shaken overnight at 310 K. The suspension was subsequently diluted 1/10 and grown for 1 h. Protein synthesis was induced for 4 h at 310 K with 1 m*M* isopropyl-β-D-thiogalactopyranoside (Boehringer Mannheim, Mannheim, Germany). Subsequent steps were performed at 277 K. The bacteria were harvested by centrifugation and resuspended in 3 ml lysis buffer (50 m*M* TRIZMA–HCl pH 8, 100 m*M* NaCl, 1 m*M* EDTA) containing 133 μM PMSF (Sigma, St Louis, MO, USA) and 266 $\mu g\ ml^{-1}$ lysozyme (Sigma) per gram of *E. coli*. Deoxycholic acid (4 mg) was added while stirring after 20 min and the mixture was kept at 310 K until it became viscous. 20 µl of 1 $mg\ ml^{-1}$ stock solution of DNAase I (Boehringer Mannheim) per gram of bacteria was then added and the mixture was kept at room temperature for 30 min. This mixture was sonicated three times for 15 s at 18 µm on ice using a Soniprep 150 (MSE, Crawley, England) and centrifuged. The supernatant was loaded onto a 2 ml prepacked glutathione Sepharose column (Pharmacia) equilibrated in lysis buffer. The column was washed with five bed volumes of lysis buffer including 1 m*M* PMSF and equilibrated with freshly prepared thrombin buffer [50 m*M* TRIZMA–HCl pH 8.0, 150 m*M* NaCl, 5 m*M* $MgCl_2$ and 1 m*M* 1,4-dithiothreitol (DTT)]. After incubation for 1 h at room temperature with thrombin buffer plus 80 units of thrombin (Sigma), cleaved *r*-grancalcin was eluted with lysis buffer plus 5 m*M* EGTA and 50 μM 4-amidinophenyl-methanesulfonylfluoride (APMSF, Sigma). Buffer was exchanged to binding buffer (100 m*M* KCl, 3 m*M* NaCl, 10 m*M* PIPES pH 7.2) plus 0.5 m*M* $CaCl_2$ and the sample was loaded onto a self-packed phenyl-Sepharose (Pharmacia) column at room temperature. Thrombin and GST eluted in the flow-through, whereas *r*-grancalcin bound to the hydrophobic matrix in the presence of calcium. The column was then washed thoroughly with binding buffer plus 0.5 m*M* $CaCl_2$. Finally, *r*-grancalcin was eluted with the binding buffer containing 5 m*M* EGTA. Fractions containing pure *r*-grancalcin were pooled and used for crystallization.

Despite the addition of protease inhibitors during purification, grancalcin becomes partially proteolysed on prolonged storage at 277 K. A second band appears on the SDS–PAGE gel in addition to full-length grancalcin. The N-terminal sequence of this new band indicated that 14 amino acids have been removed from the N-terminus of grancalcin.

2.2. Crystallization

For crystallization purposes, the protein was concentrated in a buffer containing 2 m*M* DTT, 0.5 m*M* PMSF, 1 m*M* NaN_3 and 40 m*M* HEPES pH 7.5. Crystallization was performed by the hanging-drop vapor-diffusion method at room temperature or at 291 K.

2.2.1. Apo grancalcin. The initial crystallization conditions of apo grancalcin (no Ca^{2+}) were established using the sparse-matrix method with screens I and II from Hampton Research (Laguna Niguel, CA, USA). Two crystal forms were obtained.

Form *A* crystals appeared 5 d after mixing 3 µl of 7.5 $mg\ ml^{-1}$ protein solution with 3 µl of reservoir solution containing 0.1 *M* NaAc–HAc pH 5.6, 0.1 *M* NaCl and 1 *M* $NH_4H_2PO_4$. The crystals were plate-shaped. Diffraction-quality crystals were obtained by the microcrystal selection technique (Han & Lin, 1996). The crystals grew to final dimensions of 0.5 × 0.3 × 0.3 mm in two weeks. They belong to the monoclinic system, space group $P2_1$, with unit-cell parameters a = 48.4, b = 81.1, c = 46.6 Å, β = 111.3°, contain two molecules in the asymmetric unit and diffract to 1.9 Å resolution.

Form *B* crystals were obtained using reservoir solution containing 0.1 *M* NaAc–HAc pH 4.5, 0.8 *M* $(NH_4)_2SO_4$. They were plate-shaped and grew to maximum dimensions of 0.4 × 0.4 × 0.2 mm. They are monoclinic, space group *C*2, with unit-cell parameters a = 97.0, b = 51.9, c = 75.9 Å, β = 108.5° and two molecules in the asymmetric unit. These crystals diffract to 2.4 Å resolution.

Crystals were also obtained with a well solution containing 30% poly(ethylene glycol) methyl ester (PEG–MME) 2K, 0.2 *M* $(NH_4)_2SO_4$ and 0.1 *M* NaAc pH 4.6. However, these plate-shaped crystals diffracted only to 3 Å resolution and were not further characterized.

2.2.2. Ca^{2+}-bound grancalcin. Calcium promotes aggregation of grancalcin (K. Lollike *et al.*, manuscript in preparation). In all crystallization experiments in the presence of Ca^{2+}, a precipitate was observed immediately after mixing the protein solution with the well solution. Somewhat less amorphous precipitate was observed in solutions with higher concentrations of PEGs and glycerol. All crystals grew from this precipitate. The amount of the precipitate decreased with time after crystals started to grow.

Initial crystallization conditions in the presence of calcium were obtained by screening with various molecular-weight polyethylene glycols as precipitants. The concentration of grancalcin was 6 $mg\ ml^{-1}$. 3 µl of protein solution was mixed with 3 µl of well solution to form a drop. Clusters of needle-shaped crystals appeared after 5 d from conditions containing 10–18% PEG 20K, 10–15% glycerol, 0.1 *M* HEPES pH 7.5 and 5 m*M* $CaAc_2$. Crystals could also be obtained using PEG 8K or PEG 4K as precipitant or with $CaCl_2$ instead of $CaAc_2$. The best crystals were obtained with a reservoir solution consisting of 15% PEG 20K, 15% glycerol, 50 m*M* HEPES pH 6.8 and 5 m*M* $CaCl_2$ at 291 K. The crystals grew to dimensions of 0.2 × 0.15 × 0.1 mm. They belong to monoclinic space group *C*2, with unit-cell parameters a = 97.4, b = 50.3, c = 77.6 Å, β = 108.2°, contain two molecules in the asymmetric unit and diffract to 2.5 Å resolution. Their unit-cell parameters are very similar to those of the apo form *B*. Attempts to grow these crystals at higher Ca^{2+} concentrations (10–50 m*M*) led to poorly diffracting crystals.

Crystals were also obtained using well solution containing 11% PEG 8K, 10 m*M* $CaCl_2$ and 100 m*M* MES pH 5.5. Tetragonal crystals appeared after two months and grew slowly to maximum dimensions of 0.2 × 0.2 × 0.35 mm. Unfortunately, they diffracted to less than 3.5 Å resolution at the synchrotron and showed high mosaicity.

2.3. Data collection and processing

Diffraction data for three crystal forms were collected on the ADSC Quantum 4R CCD detector at the X8C beamline, Brookhaven National Laboratory, Upton, NY. Crystals of the Ca^{2+}-bound form were flash-frozen by direct transfer from the drop to liquid nitrogen. Grancalcin crystals of Ca^{2+}-free forms were first transferred from the crystallization drop to mineral oil. The crystal was separated from the mother liquor by moving it within the oil with the help of a 0.3 mm nylon loop. The crystal was then picked up in the loop, mounted on the goniometer stand and flash-frozen in an N_2 stream at 100 K.

The indexing and data processing were performed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Crystals grown in the presence of Ca^{2+} belong to space group *C*2, with two monomers per asymmetric unit. A data set to 2.5 Å resolution was collected with an $R_{sym}(I)$ of 7.8%

and 95.9% completeness. The Ca^{2+}-free form *A* crystals belong to the $P2_1$ space group. A data set was collected to 1.9 Å resolution which was 94% complete with an $R_{sym}(I)$ of 5.3%. A data set from the Ca^{2+}-free crystal form *B* was collected to 2.4 Å resolution with a $R_{sym}(I)$ of 5.6% and 99.5% completeness. Solution of these structures is in progress.

3. Discussion

Grancalcin is a soluble protein, but it aggregates in the presence of even low concentrations of Ca^{2+}. Thus, although crystals of grancalcin in the presence of 5 m*M* $CaCl_2$ were obtained, the appearance of precipitate makes the concentration of grancalcin and Ca^{2+} in the soluble portion of the crystallization drop unknown. In order to understand the nature of conformational rearrangements triggered by calcium binding, the structures of both the Ca^{2+}-free and Ca^{2+}-bound forms of grancalcin are needed. While the apo forms crystallized rather easily, obtaining crystals in the presence of calcium was much more difficult and the crystallization process was less reproducible owing to a heavy precipitate.

It has been notoriously difficult to obtain structural information on Ca^{2+}-binding EF-hand proteins that describe both the free and bound state (Chazin, 1995; Yap *et al.*, 1999) and only a few examples are presently known. The structures of grancalcin crystallized under such different conditions should add to this limited database and provide insight into the Ca^{2+}-induced conformational changes.

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