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Crystallization and preliminary X-ray analysis of VCBP3 from *Branchiostoma floridae*

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VCBPs represent a family of proteins with highly diversified immunoglobulin-like variable regions in species thought to lack an adaptive immune system. These proteins are expected to reveal important structural and functional features that could be highly informative in projecting the evolutionary history of the adaptive immune response. Preliminary X-ray diffraction data from amphioxus (*Branchiostoma floridae)* VCBP3 crystals were collected to 2.4 Å resolution and reduced to primitive trigonal space groups $P3_{1(2)}21$. Unit-cell parameters are $a = b = 58.99$, $c = 79.21$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Two distinct crystallization conditions yielded crystals with similar morphologies and these crystals are isomorphous to each other.

1. Introduction

The adaptive immune system arose abruptly in ancestors of the jawed vertebrates approximately 500 million years ago (Litman *et al.*, 1999; van den Berg *et al.*, 2004). Proteins characteristic of adaptive immune responses [*e.g.* immunoglobulin and T-cell antigen receptor (TCR)] have been identified in all species of jawed vertebrates thus far examined (Rast *et al.*, 1997). However, no definitive homolog of either these or other genes associated with adaptive immune function has been reported in jawless vertebrates or invertebrates. Although the identity of the 'primordial' receptor that gave rise to antigen receptors in jawed vertebrates may never be established, several different families of genes that exhibit the predicted characteristics of such a receptor have been described both within and outside the jawed vertebrates (Rast & Litman, 1998).

Among the protochordates, efforts have focused on the amphioxus (*Branchiostoma floridae*), a cephalochordate that represents the most phylogenetically proximal invertebrate form on a direct line with vertebrates. Efforts to find adaptive immune genes in this species have identified a multigene family that encodes the VCBPs (variable region-containing chitin-binding proteins; Cannon *et al.*, 2002). These molecules possess two tandem V region domains as well as a chitin-binding domain (CBD) and can be classified into five major families. Comparisons of pooled mRNAs (cDNAs) (Cannon *et al.*, 2002), as well as genomic sequences derived from individual animals (Cannon *et al.*, 2004), have revealed several regions of considerable sequence substitution, *i.e.* VCBPs are diversified at both the inter- and intrafamily levels.

To determine the extent of structural similarity among the VCBP proteins, which are likely to function as innate receptors and antigen receptors characteristic of adaptive immune responses, we expressed, purified and crystallized an immunoglobulin-like variable domain from the VCBP3 protein family. An understanding of VCBP structures will provide insights into the evolutionary mechanisms that underscore the dissemination of the antigen-binding receptors.

2. Materials and methods

2.1. Constructs and expression

The amino-terminal V domain of VCBP3 was selected for the initial construct. Oligonucleotides were designed based on the VCBP3 cDNA sequence (Genbank accession No. AF520474): VCBP3-XC-F1, 5′-ATGCAGTCCATCATGACCGTCCGCA (ATG + nucleotides 49–70), and VCBP3-XC-R1, 5′-TTAGGTGTGGCCTGTCACCTTGAGCAC (TTA + antisense nucleotides 427–450). In addition to the native VCBP3 cDNA sequence, VCBP-XC-F1 included an artificial methionine codon at its 5′ end and VCBP-XC-R1 included an artificial TAA stop codon in antisense at its 5′ end. The final peptide encoded by the PCR amplicon represented the N-terminal V domain of VCBP3, *i.e.* amino acids 17–150 of Genbank No. AAN62850, beginning at the first residue after the predicted signal peptide and extending through the end of the amino-terminal immunoglobulin-like domain. PCR products were ligated into pETBlue-1 (Novagen) and sequenced for confirmation. The construct was then transformed into the *Escherichia coli* Tuner strain (Novagen) for IPTG-induced expression. 2 l cultures were

grown to OD_{600} = 0.5–0.9 at 310 K and 100 m*M* IPTG was added to a final concentration of 1 m*M*. Cultures were grown for an additional 5 h at 310 K. Induced bacterial cultures were centrifuged and stored overnight in 20% sucrose, 10 m*M* EDTA.

2.2. Refolding and purification

Thawed bacteria were brought to 200 ml in sucrose/EDTA and egg-white lysozyme (Sigma) was added at 1 mg ml^{-1} to the bacterial slurry. The slurry was processed in an EmulsiFlex C5 high-pressure homogenizer (Avestin, Ottawa, Ontario, Canada) at 69 MPa for two cycles. PMSF was added to 0.1 m*M* final concentration, 60 µl Lysonase recombinant lysozyme and 60 µl Benzonase nuclease (Novagen) were added and the homogenate was incubated at room temperature for 20 min. Centrifugation for 25 min at 15 000*g* separated the inclusion bodies from soluble components. Inclusion bodies were washed with 10 m*M* Tris pH 8.0, 5 m*M* EDTA, 0.1% Triton X-100 pH 8.0 followed by four to six alternating washes in 10 m*M* Tris and deionized water. SDS–PAGE electrophoresis confirmed that the inclusion bodies contained inducible protein in a major band of the predicted size. MALDI mass spectroscopy yielded an estimated weight of 14 886 Da (UF ICBR Protein Core), close to the predicted size of the construct (14 845 Da).

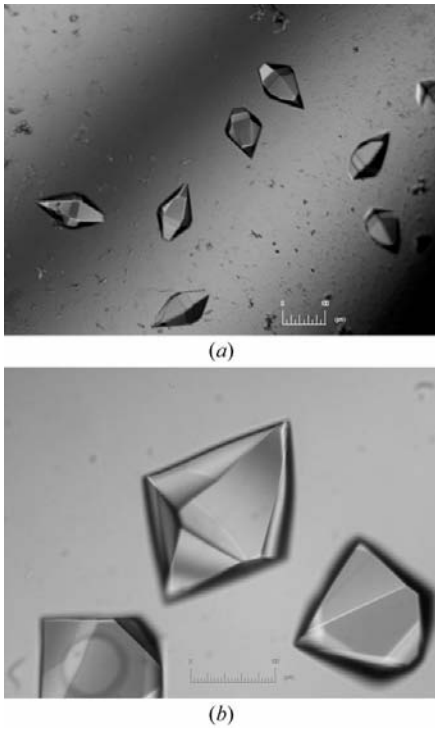

Figure 1
Formation of hexagonal VCBP3 crystals under different precipitant conditions. Scale bars are 100 µm in length. (*a*) VCBP3 crystals formed in PEG 1500, 20% glycerol. (*b*) VCBP3 crystals formed in 2.0 *M* sodium acetate, 0.1 *M* NaCl, Tris–HCl pH 7.5.

Table 1
Data-collection and reduction statistics for VCBP3 crystals.

Values for the highest resolution shell are in parentheses.

Temperature (K)	100	295
No. of frames	76	120
Crystal-to-detector distance (mm)	150	120
Observed reflections	79003	56458
Unique reflections	6574	5379
Redundancy	4	7
Resolution range (Å)	40–2.40 (2.49–2.40)	40–2.60 (2.69–2.60)
Space group	$P3_{1(2)}21$	$P3_{1(2)}21$
Unit-cell parameters	$a = b = 58.99$, $c = 79.21$	$a = b = 60.10$, $c = 80.02$
Oscillation step (°)	1	1
Mosaicity (°)	1.04	0.31
$\langle I/\sigma(I)\rangle$	13.9 (4.0)	20.2 (7.0)
Reflections >3σ (%)	75.6 (48.1)	81.9 (66.0)
Completeness (%)	99.5 (98.3)	98.3 (98.5)
$R_{\rm merge}$ (%)	10.7 (36.4)	10.7 (29.9)

Inclusion bodies were solubilized overnight at 277 K in 10 ml 7.8 *M* guanidinium–HCl, 50 m*M* Tris pH 8.0 for the inclusion bodies from 2 l of starting culture. The protein concentration was approximately 5–10 mg ml^{-1} during solubilization. Solubilized protein was centrifuged at 100 000*g* for 30 min in order to remove nucleated aggregates. The solution was added immediately to a TCEP (Pierce) disulfide-reducing agarose-gel column of bed volume 2.5 ml. The column eluate was slowly dripped (over the course of 4 h) into a large volume (~300 ml) of cold stirred refolding buffer: 0.55 *M* guanidine, 0.44 *M* L-arginine, 55 m*M* Tris pH 8.2, 21 m*M* NaCl, 0.88 m*M* KCl, 1 m*M* EDTA, 1 m*M* GSH, 1 m*M* GSSG. The cold refolding buffer was slowly brought to room temperature over 1 h in a refrigerated water bath. The 300 ml solution was dialyzed against 2 l 10 m*M* Tris, 50 m*M* NaCl pH 8.0 overnight at 277 K. The dialysate was centrifuged at 15 000*g* for 20 min and concentrated 10–25-fold in an Amicon ultrafilter with a PM10 membrane (Millipore). Clarified concentrate was then separated by FPLC on a Superdex 75 column (Amersham Pharmacia) and the appropriate size fraction (15 kDa) collected as the final purification step. Final recovery of purified VCBP3 was approximately 10 mg protein per litre of starting culture.

2.3. Crystallization

Crystals of VCBP3 were grown by the vapor-diffusion method in hanging drops (McPherson, 1999). 2 µl protein solution (10 mg ml^{-1} in 10 m*M* Tris pH 8.0, 50 m*M* NaCl) and 2 µl reservoir solution were mixed on siliconized slides and allowed to equilibrate against 1 ml reservoir solution. Three commercially available sparse-matrix crystallization (Jancarik & Kim, 1991) kits from Hampton Research were used for screening: Crystal Screen 1, Crystal Screen 2 and Crystal Cryoscreen (144 conditions). Initial screening at 291 K revealed that VCBP3 crystals formed in conditions containing different precipitants (Fig. 1). VCBP3 crystals with dimensions of 0.15 × 0.15 × 0.05 mm formed in 1.2–1.4 *M* ammonium sulfate, 0.1 *M* NaCl, 0.1 *M* HEPES pH 8.5, 12% glycerol. A set of optimization experiments found distinct conditions that produced crystals of VCBP3 over a wide range of precipitant concentration (1.6–2.6 *M* sodium acetate, 0.1 *M* NaCl, Tris–HCl pH 7.5). Smaller crystals also grew in PEG 1500, 20% glycerol. Crystallization trials at 277 K yielded similar results.

2.4. Data collection and processing

Diffraction data were collected using an R-AXIS IV^{++} 100 image-plate detector (300 × 300 mm). Crystals were mounted in glass capillaries for room-temperature data collection or on a nylon-fiber loop and flash-frozen in a nitrogen-gas stream employing an Oxford cryosystem. X-rays were generated by a Rigaku rotating copper anode with Osmic mirrors and a 0.3 mm collimator running at 40 kV and 100 mA. Data were collected using oscillation angles of 1° per frame. Each frame was exposed for 5 min.

Intensities were indexed and integrated with *DENZO* and reduced with *SCALEPACK* (Otwinowski & Minor, 1997). The molecular weight of the crystallized polypeptide was based on mass-spectrometry analysis and unit-cell contents were predicted with the *Matthews Probability Calculator* (Kantardjieff & Rupp, 2003). Data-processing statistics for two native data sets from single crystals (*i.e.* one grown using the ammonium sulfate conditions with

12% glycerol and another using sodium acetate) are summarized in Table 1. This low concentration of glycerol was able to cryoprotect VCBP3 crystals during data collection.

3. Results and discussion

Crystals grew in 3 d and nucleation in several drops could be observed immediately after setting up the crystallization experiment. Crystals with a diffraction limit of 2.4 Å and approximate dimensions of 0.15 × 0.15 × 0.05 mm were obtained in 1.3 *M* ammonium sulfate, 0.1 *M* NaCl, 0.1 *M* HEPES pH 8.5, 12% glycerol. A single cryocooled crystal of this size provided the data reported in Table 1. A crystal of similar size grown in 2.0 *M* sodium acetate, 0.1 *M* NaCl, Tris–HCl pH 7.5 was mounted in a glass capillary at room temperature and diffraction data to 2.6 Å resolution were collected with statistics of comparable quality (Table 1). Data from VCBP3 crystals grown in sodium acetate are consistent with the same space group as VCBP3 crystals grown in ammonium sulfate, $P3_{1(2)}21$, with similar unit-cell parameters.

The Matthews coefficient was used to estimate the solvent content and number of molecules in the asymmetric unit (Matthews, 1968; Kantardjieff & Rupp, 2003). In their recent re-evaluation of V_M values from more than 10 000 protein crystal forms, Kantardjieff and Rupp obtained improved distribution curves that also account for diffracting resolution limit. The sample size in their statistical analyses is more than 45 times larger than that available to Matthews in 1976 (Matthews, 1976). According to a unit-cell volume of 238 708.3 Å^3 and a molecular weight of 14 886 Da, a single molecule of VCBP3 is unambiguously most probable in the asymmetric unit. The expected solvent content is 53.98% and corresponds to a V_M value of 2.67 Å^3 Da^{-1}. Systematic absences are clearly consistent with screw axes of 2/3 or 1/3 unit-cell translations.

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