FOR THE RECORD

Crystallization and preliminary X-ray crystallographic studies on Type III antifreeze protein

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Abstract: Type III antifreeze protein, more specifically the recombinant QAE-Sephadex-binding isoform, has been crystallized in 50–55% saturated ammonium sulfate, 0.1 M sodium acetate, pH 4.0–4.5. The resultant crystals belong to the orthorhombic space group $P2_12_12_1$ with $a = 32.60$ Å, $b = 39.00$ Å, and $c = 46.57$ Å and diffract to at least 1.7 Å. A set of 1.7-Å native data has been collected, with completeness 93.4% and R_{sym} of 0.069. Initial screening for heavy-atom derivatives has yielded a Pt-bound derivative.

Keywords: antifreeze protein; crystallization; X-ray crystallography

Antifreeze proteins (AFPs) are proteins that adsorb to and inhibit the growth of ice crystals (Raymond & DeVries, 1977). AFP adsorption is believed to occur through a hydrogen-bonding match to the ice lattice (DeVries, 1984) that restricts ice growth to regions between the bound proteins (Knight et al., 1991). When there is extensive coverage of the surface by AFPs, the ice fronts are forced to grow with a local curvature that raises the surface free energy and thereby lowers the local (nonequilibrium) freezing point (DeVries & Cheng, 1992). AFPs from fish have been classified into four types (Davies & Hew, 1990). The extended AFPs (Type I and antifreeze glycoprotein) have repetitive secondary structures that have suggested hydrogen-bonding matches to prism or pyramidal planes (Wen & Laurson, 1992; Knight et al., 1993). Sequence repeats are not present in the globular AFPs (Types II and III). Recently, the protein fold of a 66-amino acid Type III AFP was deduced by 2D NMR (Sönnichsen et al., 1993). In this QAE-Sephadex-binding isoform, nine β -strands are paired to form two triple-stranded antiparallel sheets and one double-stranded antiparallel sheet, with the two triple sheets arranged as an orthogonal β -sandwich. Although this protein fold lacks repetitive motifs, the structural data were invaluable, together with an alignment of numerous Type III AFP isoform sequences (Chao et al., 1993), in suggest-

ing the location of the ice-binding site. This was confirmed by site-directed mutagenesis, which showed that three hydrophilic residues (N14, T18, and Q44) have a major role in ice binding (Chao et al., 1994). An extensive analysis of surface-accessible residues has revealed the direct involvement of a fourth amino acid side chain (C.I. DeLuca et al., unpubl.). One of the main objectives in undertaking X-ray crystallographic analysis of this AFP is to define the precise 3D arrangement of these ice-binding side chains in search of a match to the ice lattice and an explanation for the virtually irreversible binding of this protein to ice. At the same time, a high-resolution structure should also permit surface water molecules to be located, and their interaction with the protein might provide new insights into ice binding. In addition, it is important to establish the conformation of the loop region between residues 27 and 42 and its contribution to the stability of the protein fold.

Crystallization of a Type III AFP from ocean pout serum has been reported recently (Xue et al., 1994). Although the sequence of the AFP was not identified, its elution position from reversed-phase HPLC suggested that it may be one of the SP-Sephadex-binding isoforms. These variants share only 50% sequence identity with the recombinant QAE-Sephadex-binding isoform studied here. They have a much higher pI (10.3 as compared to 6.7) and are more soluble than the QAE isoform.

Recombinant Type III AFP was overexpressed and purified from inclusion bodies as described previously (Chao et al., 1994). A saturated solution of the protein was prepared by dissolving lyophilized AFP in 10 mM Tris-HCl (pH 9.0). Standard sparse matrix crystal screening (Jancarik & Kim, 1991) was first carried out, and useful preliminary crystallization conditions were obtained. Because of the low solubility of the recombinant AFP, it was crucial to work with a saturated protein solution. Diffraction-quality crystals were grown by vapor diffusion using the hanging drop method. Hanging drops contained a mixture of saturated protein solution (10 μ L) and well solution (10 μ L). The latter was composed of 50–55% saturated ammonium sulfate in 0.1 M sodium acetate buffer (pH 4.0–4.5). The concentration of protein in the saturated solution was approximately 6–7 mg/mL as determined by UV absorbance. Crystals appeared within 2 days at 20 °C and grew to a maximum size of 0.75 $\times$ 0.25 $\times$ 0.20 mm in about 2 weeks (Fig. 1).

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Fig. 1. Crystals of the AFP protein.

Diffraction data were collected at room temperature using a Marresearch Imaging Plate system (18 cm diameter) with CuK α radiation. The Rigaku RU200 generator was operated at 60 kV, 70 mA. Crystals diffracted to better than 1.7 Å, and a set of native data was collected to 1.7 Å resolution. A total 90° scan was carried out, with 2° oscillation and 30 min exposure per frame. The crystal was not sensitive to the X-ray exposure, and no significant decay was observed during data collection. There

were 6,510 unique reflections in the 1.7-Å data set, which corresponds to 93.4% completeness in the resolution range of 30–1.7 Å with R_{sym} of 0.069. The unit cell parameters were determined using DENZO (Otwinowski, 1993), and the space group was deduced from the diffraction pattern. The space group is $P2_12_12_1$, with cell dimensions: $a = 32.60$ Å, $b = 39.00$ Å, $c = 46.57$ Å. The space group was also confirmed by the unique Patterson solution for the platinum derivative in $P2_12_12_1$. Given a calculated molecular weight of ~7,018 Da and four asymmetric units in the unit cell, the Matthews coefficient (Matthews, 1968) is 2.11 Å³/Da, which is consistent with the presence of a single molecule in the asymmetric unit and an estimated solvent content of ~39%. Data frames were individually indexed and integrated using DENZO (Otwinowski, 1993), and then all frames were scaled and merged together using SCALEPACK in the DENZO (Otwinowski, 1993) package.

An extensive molecular replacement attempt has been made using X-PLOR (Brünger, 1992) and AMoRe (Navaza, 1994). The search model was the NMR structure of the same AFP protein (Sönnichsen et al., 1993). A wide range of conditions has been tested, including data resolution, Patterson radius, and various probing models with or without the loop regions that are less well defined in the NMR structures. So far, molecular replacement trials have been unsuccessful.

Initial heavy-atom screening was conducted in soaking experiments with heavy-atom solutions in 55% ammonium sulfate in 0.1 M sodium acetate buffer (pH 4.3). The crystal was soaked in 1 mM potassium platinocyanide ($K_2Pt(CN)_4$) solution for 12 h. At concentrations higher than 1 mM, $K_2Pt(CN)_4$ cracked the AFP crystals within a few minutes to a few hours. Difference Patterson synthesis calculated using the CCP4 package (1994) has indicated a Pt-binding site in the protein (Fig. 2). Unfortunately, the binding of Pt caused more than a 2% cell dimension change in the c axis, making the derivative unusable for SIR phasing beyond ~4 Å. More heavy-atom screenings are being carried out, but so far no other suitable derivatives have been found. In order to increase the chances of obtaining another heavy-atom derivative and facilitate the structure determination, we are currently preparing a number of site-specific mutants in which a surface-exposed residue is replaced by cysteine that is not otherwise present in the recombinant AFP.

Fig. 2. Potassium platinocyanide derivative difference Patterson, 10–4 Å. The map starts at the 2σ contour level above the mean density, with a contour interval of 0.25σ . **A:** u section, 0.5 Harker plane. **B:** v section, 0.5 Harker plane. **C:** w section, 0.5 Harker plane.

Once the protein X-ray structure has been solved at high resolution, it should be feasible to crystallize and solve the structures of various ice-binding mutants like T18N, in which a single side-chain replacement causes a 90% loss of antifreeze activity (Chao et al., 1994). Such comparisons between mutant and wild-type AFPs might help explain the mechanism by which this protein binds specifically and tightly to ice.

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References

- Brünger AT. 1992. *X-PLOR manual, version 3.1*. New Haven, Connecticut: Yale University.
- CCP4. 1994. Collaborative Computational Project, Number 4. The CCP4 suite: Programs for protein crystallography. *Acta Crystallogr D* 50: 760–763.
- Chao H, Davies PL, Sykes BD, Sönnichsen FD. 1993. Use of proline mutants to help solve the NMR solution structure of Type III antifreeze protein. *Protein Sci* 2:1411–1428.
- Chao H, Sönnichsen FD, DeLuca CI, Sykes BD, Davies PL. 1994. Structure–function relationship in the globular Type III antifreeze protein: Identification of a cluster of surface residues required for binding to ice. *Protein Sci* 3:1760–1769.
- Davies PL, Hew CL. 1990. Biochemistry of fish antifreeze proteins. *FASEB J* 4:2460–2467.
- DeVries AL. 1984. Role of glycopeptides and peptides in inhibition of crystallization of water in polar fishes. *Philos Trans R Soc Lond B* 304: 575–588.
- DeVries AL, Cheng CHC. 1992. The role of antifreeze glycopeptides and peptides in the survival of cold-water fishes. In: Somero et al., eds. *Water and life*. Berlin/Heidelberg: Springer-Verlag. pp 301–315.
- Jancarik J, Kim SH. 1991. Sparse matrix sampling: A screening method for crystallization of proteins. *J Appl Crystallogr* 24:409–411.
- Knight CA, Cheng CC, DeVries AL. 1991. Adsorption of α -helical antifreeze peptides on specific ice crystal surface planes. *Biophys J* 59:409–418.
- Knight CA, Driggers E, DeVries AL. 1993. Adsorption to ice of fish antifreeze glycopeptides 7 and 8. *Biophys J* 64:252–259.
- Matthews BW. 1968. Solvent content of protein crystals. *J Mol Biol* 33:491–497.
- Navaza J. 1994. AMoRe: An automated package for molecular replacement. *Acta Crystallogr A* 50:157–163.
- Otwinowski Z. 1993. In: Sawyer L, Issacs N, Bailey S, eds. *Data collection and processing*. Warrington, UK: SERC Daresbury Laboratory. pp 56–62.
- Raymond JA, DeVries AL. 1977. Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *Proc Natl Acad Sci USA* 74:2589–2593.
- Sönnichsen FD, Sykes BD, Chao H, Davies PL. 1993. The nonhelical structure of antifreeze protein Type III. *Science* 259:1154–1157.
- Wen D, Laursen RA. 1992. A model for binding of an antifreeze polypeptide to ice. *Biophys J* 63:1659–1662.
- Xue QY, Sicheri F, Ala P, Hew CL, Yang DSC. 1994. Single crystals of a Type III antifreeze polypeptide from ocean pout. *J Mol Biol* 237:351–352.