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Structure of type III antifreeze protein at 277 K

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Abstract

Fish antifreeze proteins (AFP's) depress the freezing point of blood and other body fluids below that of the surrounding seawater by binding to and inhibiting the growth of seed ice crystals. The high-resolution crystal structure of type III AFP, determined at room temperature, reveals a remarkably flat surface containing most of the ice-binding residues [Jia *et al.* (1996). *Nature (London)*, **384**, 285–288]. Since AFP's function at temperatures close to 273 K, it is important to know whether the structure determined at room temperature undergoes any change at much lower temperature. Therefore, type III AFP has been crystallized at 277 K and its structure determined. Although crystallization conditions at 277 K were similar to those at ~295 K, crystal growth took much longer at the lower temperature. Crystals grown at the two temperatures were isomorphous. Initial crystals appeared within 40–50 d and grew to their final size in about 8–12 months, instead of a couple of days at ~295 K. The type III antifreeze protein structure from crystals grown at 277 K was essentially the same as that determined at ~295 K, with the exception of some minor changes in side-chain conformation. The result is an indication that temperature has a minimal effect on the structure of type III AFP, thus lending increased physiological validity to the room-temperature structure which was used for the initial ice-binding modelling.

1. Introduction

Many organisms ranging from fish to bacteria produce proteins that allow them to survive at ambient temperatures below the colligative freezing point of their body fluids (Davies & Hew, 1990; Griffith & Ewart, 1995). These antifreeze proteins (AFP's) have the unique ability to adsorb to ice and inhibit the growth of seed ice crystals that form in solution at or below the equilibrium freezing point (Raymond & DeVries, 1977). As the solution is cooled further, there comes a point where ice crystal growth is reinitiated. The difference in temperature (K) between this non-equilibrium freezing point and the melting temperature is referred to as thermal hysteresis, the magnitude of which is a function of AFP concentration.

In polar fish, there are now five types of AFP's that have been biochemically characterized (Davies & Hew, 1990; DeVries, 1984): antifreeze glycoproteins (AFGP) and AFP types I, II, III and IV. The AFGP and type I AFP are linear repetitive structures, whereas type II and III AFP's are compact globular proteins, and type IV AFP is presumed to be a helix bundle (Deng *et al.*, 1997). Recently, the high-resolution (1.25 Å) crystal structure of recombinant type III AFP (QAE isoform) from eel pout was determined by X-ray crystallography (Jia *et al.*, 1996). This structure gave the first detailed picture of a globular antifreeze protein and revealed a remarkably flat amphipathic ice-binding site where five hydrogen-bonding atoms 4.5 Å apart match two ranks of O atoms on the {100} ice prism plane in the ⟨0001⟩ direction, and contribute to high ice-binding affinity and specificity.

Previous studies using NMR and site-directed mutagenesis (Sönnichsen *et al.*, 1993; Chao *et al.*, 1994) identified four key ice-binding residues, Gln9, Thr18, Gln44, Asn14. The recent X-ray structure (Jia *et al.*, 1996) further identified Thr15 and the carbonyl O atom of Ala16 (A16O′) to be involved in ice binding. Residues Gln9, Thr15, Gln44, Thr18 and A16O′ form a flat plane which presents the hydrogen-bonding groups for interaction with the prism face of ice. The mutation of any one of the ice-binding residues (Gln9, Asn14, Thr18, Gln44 and Thr15) caused altered antifreeze activity, which could be rationalized based on changes observed in the mutant crystal structures (DeLuca, 1997). The ice-binding atoms in the protein form a pseudo-parallelogram with Thr18, A16O′ and Gln44 on one side, and Gln9, Thr15 and Asn14 on the other. The distance of 4.5 Å that separates these two sets of parallel groups matches the spacing of O atoms on the prism plane {10$\bar{1}$0} in the direction ⟨0001⟩ (Jia *et al.*, 1996).

Previously, the crystals of type III AFP were grown at room temperature (~295 K) and the data were also collected at ~295 K (Jia *et al.*, 1995). Since AFP's can only bind to ice when seed ice crystals are present, room temperature is more than 22 K above the physiological temperature at which AFP's function. An issue of legitimate concern is whether or not a structure determined from crystals grown at room temperature can represent a model for AFP's at physiological temperatures. NMR approaches have been used in an attempt to address this problem by collecting solution structure data from AFP's close to the freezing point (Chao *et al.*, 1994; Gronwald *et al.*, 1996). No major structural perturbations were observed at low temperatures. As for the crystal structure, nothing was previously known about the effects of temperature. This subject should be of general interest to structural biologists because it compares the X-ray structure of the same protein crystallized at two different temperatures. In addition, crystal structure determination at temperatures close to the freezing point bears significance in characterizing cold-adapted proteins such as AFP's. Furthermore, the precise match between the regularly spaced ice-binding residues and the ice lattice demonstrated the crucial importance of the side-chain conformation of these residues (Jia *et al.*, 1996). Hence, we were not only interested in determining the overall fold but also the detailed side-chain conformation of the AFP at 277 K.

In this communication, we report the structure determination of type III AFP from crystals grown at 277 K and its comparison to the room-temperature structure. The resultant 277 K structure is essentially the same as that at ~295 K.

2. Materials and methods

2.1. *Expression and purification*

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 m*M* Tris–HCl (pH 9.0).

2.2. *Crystallization*

The protein was crystallized by the hanging-drop vapour-diffusion method using ammonium sulfate as precipitant. The initial protein solution at a concentration of 6 mg ml^{-1} was mixed with an equal volume of a reservoir solution containing 53–55% ammonium sulfate, which was buffered with 0.1 *M* sodium acetate (pH 4.5) at 277 K. These conditions are similar to those used for room-temperature crystallization (Jia *et al.*, 1995). Crystallization plates were placed in a 277 K cold room and at no time were crystals exposed to room temperature. Crystal observation and manipulation were performed in the cold room, and crystals were transported on ice from the cold room to the X-ray facility for data collection under cryo-cooling conditions (277 K).

2.3. *X-ray data collection and structure refinement*

Diffraction data were collected using a MAR Research imaging plate equipped with a Rigaku rotating-anode generator. The crystal was mounted in a capillary tube and data collection was carried out at 277 K using a cryo-cooling apparatus (Oxford Cryosystems Cryostream, Oxford, UK) to maintain the same temperature as crystallization. The crystals diffracted to 1.65 Å or better and a set of data was collected to 1.65 Å resolution. This is comparable to the diffraction of the room-temperature crystals, although at a synchrotron source they diffracted to 1.25 Å resolution. A total 100° scan was carried out, with 1° oscillation and 200 s exposure per frame. Data were processed and scaled with the *DENZO* package (Otwinowski, 1993). The room-temperature structure (Jia *et al.*, 1996) was used for refinement against the 277 K data, using *X-PLOR* (Brünger, 1993).

3. Results and discussion

Under cold-room temperature crystals appeared within 40–50 d but took 8–12 months to grow to their final size of about 0.25 × 0.15 × 0.1 mm. The growth of type III AFP crystals at 277 K was substantially slower than that at ~295 K where crystals generally formed within 2 d (Jia *et al.*, 1995). It is apparent that the rate of nucleation and crystal growth of type III AFP decreased substantially at lower temperature. Nevertheless, after a long period of time reasonable-sized crystals did finally form at 277 K. Similar to the room-temperature crystals, the 277 K crystals were stable to X-ray radiation and no significant decay was observed during data collection. However, upon completion of the data collection, the crystal in the capillary tube melted completely once the 277 K cryostream was stopped. The 277 K crystal belongs to the space group $P2_12_12_1$ with unit-cell parameters a = 32.69, b = 38.84, c = 47.51 Å, which is isomorphous to the room-temperature crystal. Refinement using *X-PLOR* (Brünger, 1993) went smoothly, and water molecules were gradually introduced into the model, giving a final R of 0.199 and $R_{\rm free}$ of 0.282, respectively. Model quality assessment using *PROCHECK* (Laskowski *et al.*, 1993) demonstrated the excellent geometry of the 277 K structure, with 96.4% of residues in the most favoured region of a Ramchandran plot, 3.6% in additional allowed regions and no residue residing in the generously allowed or disallowed regions. The room-temperature structure had identical statistics. Final reflection data and refinement statistics of the 277 K structure, along with the comparison to the room-temperature structure, are summarized in Table 1. Since the 277 K structure is of lower resolution, only 45 water molecules were placed into the structure as compared with the 90 water molecules placed in the ~295 K structure (Jia *et al.*, 1996). Similar to the room-temperature crystal structure, the water molecules in the 277 K structure showed no regular pattern that might resemble an ice lattice.

Table 1. *Data collection and refinement statistics of the 277 K structure compared with the room-temperature structure*

Temperature (K)	277	295†
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions (Å)		
a	32.69	32.33
b	38.84	38.98
c	47.51	47.48
Solvent content (%)	~39	~39
Resolution ranges (Å)	50.00–1.65	50.00–1.25
Total No. of reflections	27105	46873
No. of unique reflections	6660	16529
$R_{\rm sym}$	0.061	0.049
Completeness (%)	86.6	96.6
Refinement		
Resolution ranges (Å)	8.0–1.65	8.0–1.25
R ($R_{\rm free}$, 5%)	0.199 (0.282)	0.198 (0.225)
No. of protein atoms	482	482
No. of water atoms	45	90
R.m.s.d. of bond lengths (Å)	0.019	0.014
R.m.s.d. of bond angles (°)	2.72	2.90

† Data taken from Jia *et al.* (1996).

Structural comparison was carried out using *LSQKAB* in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The backbone and side-chain r.m.s. deviations between the room-temperature and 277 K structures are 0.258 and 0.594 Å, respectively. An overlap of the type III AFP structures crystallized at ~295 K and 277 K is depicted in Fig. 1. It is apparent that the two structures are essentially identical with minimal structural change. As shown in Fig. 1, type III AFP exhibits a compact folding architecture. Although there are only six short irregular β-strands and a one-turn α-helix, many β-like inter-residue hydrogen bonds exist to give rise to a very stable scaffold. These features may well help the protein resist cold denaturation. A few residues such as Ser24, Glu25, Thr28, Glu35, Arg39 and Leu55 that are located away from the ice-binding site, along with the N- and C-termini, show some variation in the structure crystallized at 277 K, but these areas also tend to be flexible in the structure crystallized at ~295 K. In addition, there is a small main-chain shift in the loop region

between Pro12 and Leu17 with a maximum displacement of 0.68 Å occurring at the Thr15 Cα atom. One interesting observation concerns Lys61, which in the ~295 K structure is hydrogen bonded to the two key ice-binding residues, Asn14 and Gln44. In the 277 K structure, Lys61 appears to make an additional hydrogen bond to the carbonyl O atom of Val45 which may contribute to the main-chain shift in the Pro12–Leu17 loop region described above.

In spite of some small differences such as Lys61 and the number of waters placed, the two protein structures are very close, demonstrating that the type III AFP structure does not undergo any large changes in protein fold or conformation as it approaches the freezing point. This was not surprising given that the isomorphous crystals were obtained from the two temperatures. This result is also in agreement with the NMR studies that suggested that there were no extensive conformational changes in AFP's upon decreasing the temperature to close to the freezing point (Chao *et al.*, 1994; Gronwald *et al.*, 1996). More importantly, the side-chain conformation of the key ice-binding residues effectively remains unchanged at 277 K. It was shown that a precise match between the ice-binding residues and the ice lattice was one of the crucial factors that allow the AFP to bind to ice very tightly (Jia *et al.*, 1996). This was further demonstrated by the mutant crystal structures. Large perturbation at the ice-binding site pronouncedly affected the ice-binding properties of the AFP (DeLuca, 1997). The 277 K crystal structure not only demonstrated that type III AFP's overall structure was not affected by the crystallization temperature but also revealed that the side-chain conformation of the ice-binding residues are almost identical to that of the room-temperature crystal structure. It should be noted that the fact that nucleation and growth of crystals are slowed down considerably implies that there is no structural memory of the 295 K state involved (*e.g.* pre-formed nuclei at 295 K) but we are looking at authentic low-temperature states. Since currently we are not able to crystallize type III AFP at even lower temperature such as below 273 K and visualize ice binding onto the AFP directly, we feel strongly that the 277 K study is a important step forward in this direction. It certainly provides significant support to the room-temperature structure and its ice-docking model. Crystallization of proteins at different temperatures (room temperature and close to physiological temperature) is important for proteins that function at unusual temperatures including AFP's and some thermostable proteins such as Taq polymerase. This knowledge will certainly contribute to our understanding of protein structure–function relationships in general. As demonstrated here, X-ray crystallography, which is usually restricted by various crystallization constraints, can also play an important role in structural characterization at varying temperatures.

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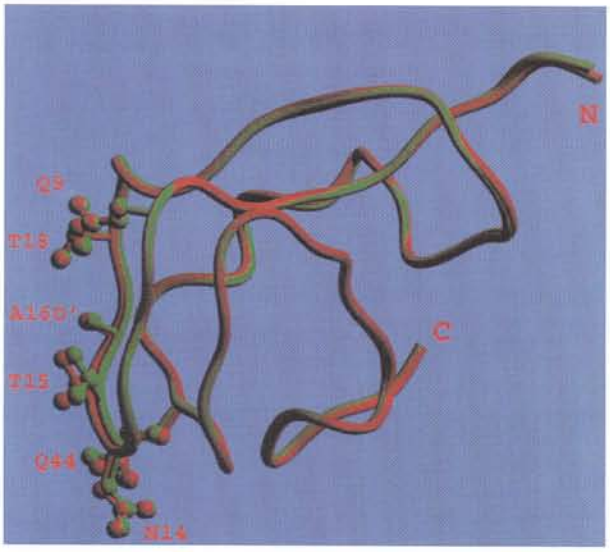

Fig. 1. Ribbon diagram of the 277 K (red) and ~295 K (green) structures, with their main chains overlapped using *LSQKAB* in *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Ice-binding residues are illustrated with the ball-and-stick model. The diagram was produced using *SETOR* (Evans, 1993).

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