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Crystallization and preliminary X-ray analysis of insect antifreeze protein from the beetle *Tenebrio molitor*

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Hyperactive antifreeze protein from the beetle *Tenebrio molitor* (TmAFP) was produced in *Escherichia coli* and purified by gel-permeation chromatography and HPLC. An iodinated derivative was prepared by incubating the 8.5 kDa TmAFP with *N*-iodosuccinimide. Native and iodinated TmAFP produced two different crystal forms when crystallized using the hanging-drop vapor-diffusion technique. Native crystals were rectangular plates that diffracted to ~2.5 Å resolution. They were monoclinic and belonged to the space group $P2_1$, with unit-cell dimensions $a = 38.4$, $b = 73.4$, $c = 59.3$ Å, $\beta = 97.0^\circ$. Crystals of iodinated TmAFP formed elongated hexagons that allowed data to be collected to ~1.4 Å. These crystals belonged to the space group $P6_1$ (or $P6_5$), with unit-cell dimensions $a = 73.85$, $b = 73.85$, $c = 53.15$ Å. There were two molecules per asymmetric unit, which corresponds to $V_m = 2.46$ Å Da^{-1} and 51% solvent content. A twofold non-crystallographic symmetry was evident from self-rotation calculations.

1. Introduction

One of the strategies that certain 'cold-blooded' organisms use to guard against freezing at sub-zero temperatures is the synthesis of antifreeze proteins (AFP), also known in the insect literature as thermal hysteresis proteins (THP). It is generally accepted that these macromolecular antifreezes inhibit ice growth by binding to the ice surface in an adsorption–inhibition mechanism, which results in a non-colligative depression of the freezing point below the melting point (Raymond & DeVries, 1977; Knight *et al.*, 1991).

AFPs have been isolated from a variety of organisms ranging from marine fishes to bacteria (Davies & Sykes, 1997; Yeh & Feeney, 1996; Ewart *et al.*, 1999). Recently, AFPs have been isolated and characterized from three insect species: a moth (*Choristoneura fumiferana*, spruce budworm; Tyshenko *et al.*, 1997) and two beetles, *T. molitor* (common yellow mealworm; Graham *et al.*, 1997) and *Dendroides canadensis* (Duman *et al.*, 1998). These insect AFPs are remarkably active and at low protein concentrations their specific activities are 10 to 100 times greater than those of fish AFPs (Graham *et al.*, 1997; Tyshenko *et al.*, 1997). *T. molitor* and *D. canadensis* AFPs share 40–66% amino-acid identity. The moth and beetle AFPs are unrelated but have some features in common. They are both small proteins (8–9 kDa), extensively disulfide bonded, with amino-acid compositions rich in threonine.

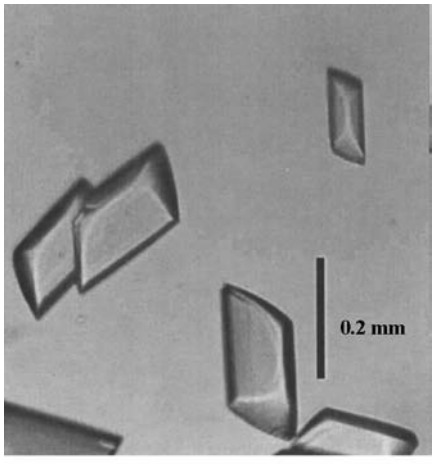
Recently, numerous AFP cDNAs from *T. molitor* were cloned and sequenced (Liou *et al.*, 1999). They encode isoforms containing 84, 96 or 120 amino acids that are largely made up of tandem 12 amino-acid repeats of consensus sequence CT*x*S*xx*C*xx*A*x*T. We previously expressed one of the 84 amino-acid isoforms containing five 12 amino-acid repeats in order to confirm its identity as an AFP (Graham *et al.*, 1997). The production of this recombinant protein in milligram quantities has opened the way to its structural characterization.

So far, the structures of different fish AFPs are proving to be remarkably diverse (Davies & Sykes, 1997). AFP I is a long single α-helix (Sicheri & Yang, 1995), AFP II has a typical C-type lectin fold (Gronwald *et al.*, 1998), AFP III is a globular molecule, but with some flat surfaces (Jia *et al.*, 1996), and AFP IV is predicted to be a helix bundle (Deng & Laursen, 1998). Despite this diversity, there is general acceptance that one surface on the AFP is responsible for the ice-lattice matching. This matching is easier to conceptualize with repetitive AFP, but so far only two of the five fish AFPs (antifreeze glycoproteins and type I) and two of the three insect AFPs (beetle AFPs) have repetitive structures. Recently, the disulfide-bond mapping of *D. canadensis* AFP was established (Li *et al.*, 1998). Given the pattern of 12 amino-acid repeats in its primary structure with regularly spaced Cys, we have proposed an initial model for *T. molitor* AFP (TmAFP) where it could fold into a β-helix structure with regularly spaced disulfide bonds in each repeat. In this model, nearly all the Thr

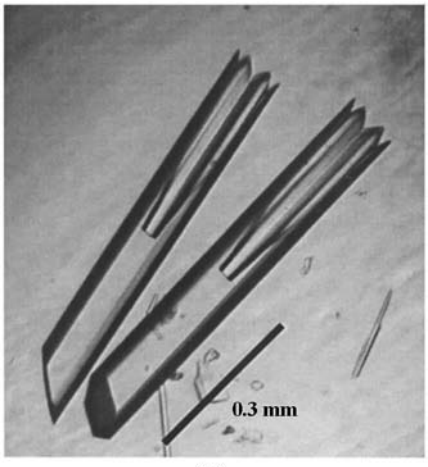
residues would be aligned on one side of the protein to present a potential ice-binding region (Liou *et al.*, 1999). Solving the TmAFP structure will provide the definitive solution to how such an extremely regular sequence makes up a three-dimensional structure. It should also provide the structural basis for the greater thermal hysteresis activity of this insect AFP compared with fish AFPs.

2. Protein purification and iodination

Recombinant TmAFP was expressed in *E. coli* and purified (Liou *et al.*, unpublished work). To iodinate TmAFP, HPLC-purified protein (2 mg ml^{-1}) was incubated with 10 m*M* *N*-iodosuccinimide in 50 m*M* sodium acetate (pH 3.7) at room temperature (295 K) for 2 h. The iodinated protein was then purified by reverse-phase HPLC on a C-18 semi-preparative column using a linear gradient of acetonitrile (buffer *B*) in 0.1% TFA (buffer *A*). The modified TmAFP eluted from the column at around 25–26% acetonitrile, compared with 24% for the wild type. As measured by MALDI mass spectrometry, the mass of iodinated TmAFP was 260.0 Da heavier than that of the unmodified protein. Since there is only one tyrosine in TmAFP, this result suggested that there were two iodines bound to this residue.



(*a*)



(*b*)

Figure 1
(*a*) Native crystals of TmAFP. The approximate crystal size is 0.2 × 0.1 × 0.08 mm. (*b*) Iodinated crystals of TmAFP. The approximate size for the collectable region is 0.03 × 0.03 × 0.2–0.4 mm (data collection was conducted only on the solid region; the hollow tail region was sliced off).

3. Crystallization

Crystallization conditions for native TmAFP were screened using the hanging-drop vapor-diffusion method at room temperature (295 K). Drops containing 2 µl of protein (10 mg ml^{-1}) and 2 µl of mother liquor were equilibrated against 1 ml of reservoir solution. Sparse-matrix screens (Jancarik & Kim, 1991) for initial trials were performed using Hampton Research screen kits (Hampton Research, California, USA). After two weeks, clusters of very thin crystals were observed from 1.8 *M* Li_2SO_4, 100 m*M* Na HEPES buffer pH 7.5. An expansion of this leading condition was conducted over a broad range of Li_2SO_4 concentrations (1.4–2.2 *M*) and pH values between 6.0 and 9.0 (MES for pH 6.0–7.0, Na HEPES for pH 7.0–8.0 and Tris–HCl for pH 8.0–9.0). However, the resultant crystals were either needle-like clusters or small stacked plates with poorly defined surface and were not suitable for diffraction studies. When the Na HEPES or Tris–HCl buffers were replaced by glycine–NaOH, small rectangular-shaped crystals (0.02 × 0.03 × 0.01 mm) with defined edges were obtained using the hanging-drop vapor-diffusion technique, as for example with 1.5 *M* Li_2SO_4, 100 m*M* glycine–NaOH pH 9.0 and 6 mg ml^{-1} of protein, but they were still too small to be useful for X-ray diffraction analysis. A further improvement in TmAFP crystal size was achieved using serial macro-seeding techniques. Single seed crystals were washed and transferred in a capillary tube to a fresh drop that contained 1.5 *M* Li_2SO_4, 100 m*M* glycine–NaOH pH 9.25 and only 3 mg ml^{-1} protein. To prevent the growth of multi-layer crystals, the seeds had to be transferred to a fresh drop every 2–3 d, depending on the growth rate of the crystals. Within two to three weeks, the best crystals, obtained from several rounds of seeding, reached dimensions of 0.2 × 0.1 × 0.08 mm (Fig. 1*a*). Unfortunately, the tendency to grow plate-like multi-crystals was not completely overcome.

Similar crystallization conditions did not work for iodinated TmAFP and screening was subsequently carried out using the Hampton Research screen kit, which was performed at room temperature (295 K). The hanging-drop vapor-diffusion method was used to screen crystallization conditions; drops containing 1 µl of TmAFP (6 mg ml^{-1}) and 1 µl of mother liquor were equilibrated against 1 ml of reservoir solution. Needle-like crystals were observed within 2 d from a condition containing 1.8 *M* ammonium sulfate, 100 m*M* MES pH 6.5, 10 m*M* cobalt chloride. After optimization of the crystallization condition, diffraction-quality crystals of iodinated TmAFP were obtained in 1.3 *M* ammonium sulfate, 100 m*M* sodium citrate pH 5.5, 10 m*M* cobalt chloride and 10 mg ml^{-1} of protein. These crystals appeared as hexagonal cylinders and attained dimensions of about 0.03 × 0.03× 0.2–0.4 mm (solid region only; Fig. 1*b*). We also investigated the growth of native crystals using this crystallization condition, but only obtained multiple plate-like or twisted crystals.

4. X-ray diffraction analysis

Native data collection was attempted using synchrotron radiation with a Quantum IV CCD detector at the F1 station of the Cornell High Energy Synchrotron Source. To prepare for low-temperature data collection (100 K), native crystals were immersed in high-density mineral oil and a small piece of filter paper was used to remove the mother liquor from crystal surfaces. Crystals were mounted individually in loops and immediately frozen and stored in a vial containing propane. Iodinated AFP data collection was carried out at the X8C beamline of Brookhaven National Laboratory. Crystals were dipped into a solution containing 1.4 *M* ammonium sulfate, 100 m*M* sodium citrate pH 5.5, 10 m*M* cobalt chloride, 25% glycerol and were flash-frozen in the Cryostream. Data were processed using *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997).

The native crystal diffracted to ∼2.5 Å in the synchrotron source. It was readily indexed to be monoclinic, with unit-cell parameters a = 38.2, b = 72.5, c = 58.8 Å, β = 97.0°. The diffraction pattern from native crystals, however, was of poor quality. The reflection spots showed either apparent twinning or 'streaking', which was particularly severe at higher resolutions. The same observation was made with many native crystals, even though most of them appeared to be single under the microscope. In addi-

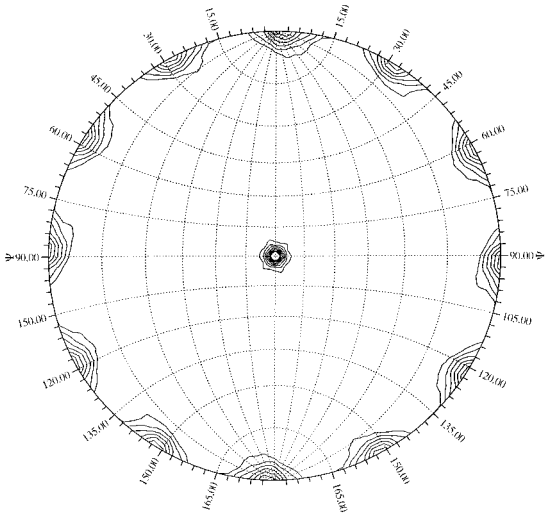


Figure 2
The self-rotation calculation at the $\kappa = 180^\circ$ section shows a single twofold non-crystallographic symmetry 30° inclined from the crystal *xy* plane. The peak is 83% of the height of the origin peak.

tion, all these crystals underwent rapid decay upon exposure to X-rays, even when frozen.

In contrast, the iodinated crystals were of excellent quality, diffracting to 1.4 Å or better. A 1.4 Å data set was collected with an overall completeness of 96.5% (95.6% for 1.45–1.4 Å shell) and an $R_{\rm sym}$ of 0.056 (0.208 for the 1.45–1.4 Å shell). There were a total of 32 557 unique reflections and the overall $I/\sigma(I)$ was 11.3 (3.8 for the 1.45–1.4 Å shell). These crystals had hexagonal space group symmetry ($P6_1$ or $P6_5$), with unit-cell parameters a = 73.85, b = 73.85, c = 53.15 Å. Given the molecular mass of TmAFP (8 510 Da), the Matthews coefficient (V_m) was 2.46 Å Da^{-1}, corresponding to two molecules per asymmetric unit and a solvent content of 51% (Matthews, 1968). A self-rotation function (Crowther, 1972) was calculated using data in the resolution range 10–2 Å to determine the non-crystallographic symmetric elements. Fig. 2 shows the self-rotation results for the $\kappa = 180^\circ$ section, clearly demonstrating the existence of a single twofold non-crystallographic symmetry 30° inclined from the crystal *xy* plane.

Evidently, the iodination approach not only offered a dramatic improvement to the problematic crystallization of native TmAFP, but also generated an excellent heavy-atom derivative. It may also be possible to extend this approach to improve the crystallization of the 9.0 kDa insect AFP from spruce budworm (Graether *et al.*, 1999) which, like that of native TmAFP, has been difficult and time-consuming. In light of the small size of TmAFP and its single Tyr residue, iodination provides a relatively large change to the protein. In this example, chemical modification by iodination has been a useful and convenient technique to provide a new crystallization target that includes a heavy atom. Consequently, iodination of TmAFP has practically opened the way for structure determination, as illustrated by the fact that an iodinated AFP III structure was solved by single anomalous scattering (SAS) facilitated by non-crystallographic symmetry averaging (Yang *et al.*, 1998). Similarly, we can take either a SAS and/or a MAD approach. Given the much improved crystallization of the iodinated TmAFP, conventional heavy-atom screening also becomes feasible.

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