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Elevated temperature and tyrosine iodination aid in the crystallization and structure determination of an antifreeze protein

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Production and refolding of recombinant *Choristoneura fumiferana* antifreeze protein (CfAFP) leads to a disulfide-bonded product containing dynamic conformational microheterogeneity. Difficulties in the crystallization of this protein arising from its microheterogeneity were overcome by screening of crystallization conditions at various temperatures and finally using a temperature of 318 K to obtain diffraction-quality crystals. In addition, heavy-atom derivatization of this protein required the iodination of a specific tyrosine residue, leading to the successful single anomalous scattering (SAS) structure determination. The techniques of higher temperature screening, to reduce dynamic conformational microheterogeneity, and defined tyrosine iodination, for specific heavy-atom incorporation, are methods which can be employed with other proteins to aid in structure determination.

1. Introduction

X-ray crystal structure determination of a protein typically requires a source of milligram to gram quantities of pure protein. Sometimes large amounts of protein can be obtained directly from a host organism, but in the case of *Choristoneura fumiferana* antifreeze protein (CfAFP) this is not possible. CfAFP is expressed in the larval stage of the insect, at which time the caterpillars are $\leq$1 mm in length, and is comprised of many isoforms which further reduces the yield of a particular isoform to microgram quantities (Tyshenko *et al.*, 1997; Doucet *et al.*, 2000). In this situation, recombinant protein expression can be used to produce greater quantities of a single isoform. However, as the native host is not typically used for recombinant protein expression, this can result in the desired protein being poorly or improperly folded. Recombinant CfAFP produced in *Escherichia coli* is a case in point and must be refolded from inclusion bodies in order to obtain active protein (Gauthier *et al.*, 1998). This process produces a final product that contains dynamic conformational microheterogeneity manifested as two interchangeable protein conformers. Here, we describe the use of heat to reduce this microheterogeneity in order produce protein crystals of CfAFP for structure determination.

Production of a recombinant protein also allows manipulation of the protein to aid in heavy-atom derivatization. Here, we discuss directed iodination of a specific tyrosine residue prior to crystallization, which is aided by our ability to mutate a second tyrosine residue in CfAFP to phenylalanine. This directed iodination resulted in a derivatized protein amenable to SAS structure determination.

2. Methods, results and discussion

2.1. Using heat to promote crystal growth through a reduction in conformational microheterogeneity

To increase the probability of obtaining crystals of a specific protein, it must be purified to homogeneity. Removing non-target proteins is becoming an increasingly routine process. However, a 'pure' target protein may still be microheterogeneous in terms of its post-translational modification (*e.g.* glycosylation and charged states) and aggregation state (Van der Laan *et al.*, 1989; Lorber & Giegé, 1992; Thomas *et al.*, 1998). This microheterogeneity, although at times difficult to detect, needs to be overcome in order to crystallize the protein of interest or to improve crystal quality. Dynamic structural or conformational microheterogeneity can also arise and frustrate crystallization attempts (McPherson, 1982). Here we describe a specific case, although generally applicable, in which conformational microheterogeneity was suspected and overcome with the use of higher temperatures for crystallization.

Diffraction-quality crystals of *C. fumiferana* (spruce budworm) antifreeze protein (CfAFP) were difficult to obtain (Leinala *et al.*, 2002). This led to re-examination of the protocol used to purify recombinant CfAFP. In the last step, C-18 reversed-phase HPLC chromatography is used to further purify CfAFP obtained as a single peak from an ion-exchange S-Sepharose column (Gauthier *et al.*, 1998). The HPLC step

results in two closely eluting CfAFP peaks, both of which display antifreeze activity. However, the activity of the more tightly bound material exhibits a higher degree of variability in antifreeze activity measurements, resulting in a larger standard deviation of values (Gauthier *et al.*, 1998). It was determined that heating of the second HPLC peak to 338 K followed by its reapplication to the HPLC C-18 column resulted in ~80% conversion of the second peak to the first peak. Also, reapplication of the purified first peak always resulted in a HPLC profile that contained a small amount of the second CfAFP peak. This suggested that two conformers of recombinant CfAFP protein were in equilibrium.

Therefore, crystallization screens were performed at a variety of temperatures in order to find one where the microheterogeneity within the protein sample could be reduced. Screening was performed at 277, 295, 303, 310, 318 and 333 K using the hanging-drop vapour-diffusion method. As the temperature increased, so did the number of conditions producing crystalline materials (Fig. 1). However, 333 K tended to cause widespread precipitation of the protein. The most favourable crystallization condition occurred at 318 K, although a finer grid screening around this temperature was not performed. In contrast, this condition did not yield crystals at 277 or 295 K. Clearly, increased temperature dramatically increased crystal size and quality.

When crystallization conditions were screened for an iodinated form of CfAFP, derivatized for structure solution as described below, high temperatures were again required for crystal growth. In this case, 24 h equilibration of the hanging drop at room temperature was required before transfer to 318 K, where crystal growth occurred. If the crystallization setup was placed directly at 318 K without prior room-temperature equilibration, this resulted in precipitation of the protein. It appears that an initial nucleation event occurred at room temperature and was followed by crystal growth at the higher temperature, where conformational microheterogeneity was again reduced. Possibly the pre-equilibration of the crystallization drop at room temperature before transfer to 318 K led to drop conditions that were optimal for the occurrence of nucleation, while immediate placement of the trials at 318 K directed the solubility of the protein too quickly through the nucleation stage, resulting in only the formation of precipitates.

Although temperature is a parameter regularly altered when screening for protein crystallization conditions, the vast majority of the entries in the Biological Macromolecule Crystallization Database (Gilliland *et al.*, 1994; McPherson, 1999) reported crystallization temperatures at 277 K or room temperature. Clearly, there is opportunity for further exploration of temperature, not only to alter protein solubility, but also to reduce structural or conformational microheterogeneity that may be found at particular temperatures for particular proteins.

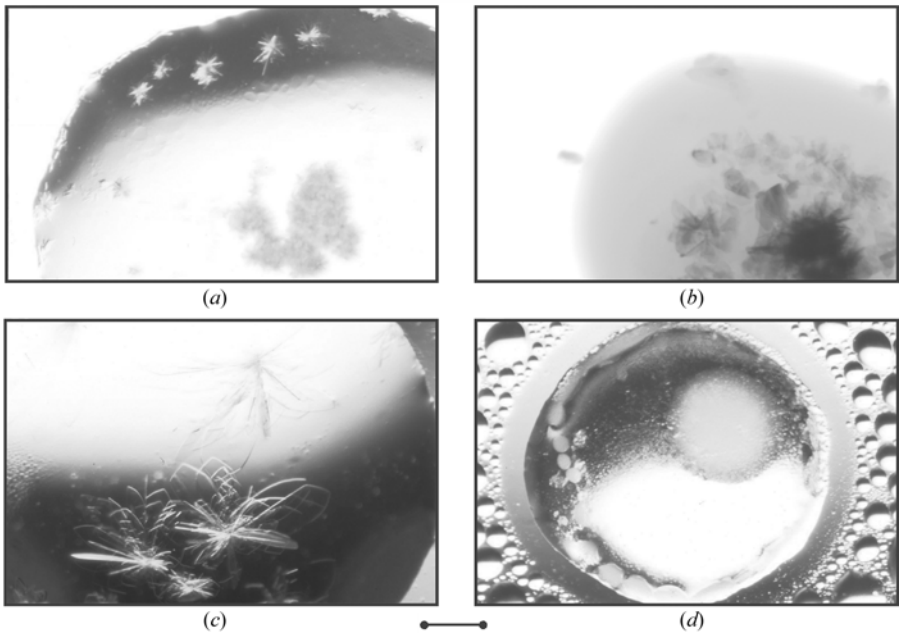

Figure 1
Crystallization of CfAFP at various temperatures using the hanging-drop vapour-diffusion method employing 20% 2-propanol, 20% PEG 4000 and 100 m*M* sodium citrate pH 5.0. (*a*) Small needle crystals at 303 K. (*b*) Larger thin plate crystals at 310 K, stained with Izit (Hampton Research) protein crystal dye. (*c*) Large thicker plate crystals at 318 K. (*d*) Protein precipitation and aggregation at 333 K. The scale bar corresponds to ~0.2 mm in (*a*), (*b*) and (*c*) and to ~0.4 mm in (*d*).

2.2. Iodination of a specific tyrosine residue for SAS

Although crystallization of native CfAFP was improved at 318 K, the crystals proved difficult to derivatize using conventional heavy-atom soaking techniques. This can be a general problem for many protein crystals. Methods involving selenomethionine incorporation (Hendrickson, 1991) or the heavy-atom labelling of substrates, inhibitors and ligands (Blundell & Johnson, 1976) have been designed to specifically derivatize macromolecules prior to crystallization. CfAFP does not contain any methionine residues and has ice as its ligand; therefore, these conventional labelling techniques could not be used.

Iodinating reagents can be used to selectively modify tyrosine residues (Glazer *et al.*, 1975; Lundblad, 1995). Even in earlier times of X-ray crystallography, iodination has been used as a marker for interpreting electron-density maps (Kretsinger, 1968; Sigler, 1970) and as a heavy-atom derivative for multiple isomorphous replacement (MIR; Matthews, 1966). Although iodine has been used on many occasions as a derivatizing compound for MIR structure solution, iodination has recently been shown to be a suitable derivative for single anomalous scattering (SAS) or single isomorphous replacement with anomalous scattering (SIRAS) phasing methods. Recently solved structures employing I-SAS or I-SIRAS include, for example, bovine neurophysin II dipeptide complex (Chen *et al.*, 1991), type III antifreeze protein from ocean pout (Yang *et al.*, 1998), acetylxylan esterase (Ghosh *et al.*, 1999), *Tenebrio molitor* antifreeze protein (Liou, Tocilj *et al.*, 2000) and CfAFP (Leinala *et al.*, 2002). A survey of the literature indicates that in the majority of cases where iodine was used in derivatizing proteins, crystals were soaked in an iodide-containing solution, using variations of the procedure described in Kretsinger (1968) and Sigler (1970), in the typical manner for heavy-atom derivatization of crystals. Screening of many conditions may be needed before a successful derivative is obtained. In contrast, specific incorporation of iodine into the protein prior to crystal-

lization will ensure full derivatization of the resulting crystals.

In the case of CfAFP, where conventional heavy-atom derivatization was not successful, direct iodination of tyrosine residues was attempted. Chemical iodination of tyrosine-containing proteins can produce a mixture of uniodinated, mono- and di-iodotyrosine residues. To reduce the heterogeneity of the final iodinated product, Tyr33, one of the two tyrosine residues in CfAFP (Tyr26 and Tyr33), was mutated to a phenylalanine (Leinala *et al.*, 2002). This single tyrosine-containing CfAFP mutant (Y33F-CfAFP) was reacted with a solution of 10 m*M* *N*-iodosuccinimide in 50 m*M* sodium acetate pH 3.7 for 2 h (Brady *et al.*, 1990) and the products were resolved by C-18 reversed-phase HPLC into the uniodinated, mono- and di-iodinated forms of the protein. Doubly iodinated CfAFP was confirmed using mass-spectrometric analysis. This iodination method is similar to the one employed with *T. molitor* antifreeze protein (Liou, Davies *et al.*, 2000) and demonstrates the feasibility and reproducibility of the method.

Doubly iodinated Y33F-CfAFP was used for crystallization screening in combination with high temperatures as described above. Crystals of the doubly iodinated CfAFP, which were not isomorphous to the wild type, were obtained (Fig. 2). SAS structure determination of CfAFP was performed on the doubly iodinated Y33F-CfAFP crystals (Leinala *et al.*, 2002). The advantage of iodine for SAS is that the $L_{\rm I}$ edge is at 2.39 Å. Although this is not very amenable for data collection at synchrotron sources, it provides sufficient anomalous scattering at the home-source Cu $K\alpha$ wavelength of 1.54 Å. Therefore, iodine SAS structure determination can be performed in-house, using a single data set, without the need to obtain data from a native protein crystal.

Upon analyzing the iodinated tyrosine residue within the CfAFP crystals, it was observed that these residues provide significant protein–protein crystal contacts in the form of di-iodotyrosine stacking (Fig. 3). Since crystals cannot be obtained in this condition with uniodinated protein, the iodination of the tyrosine residue is critical for the formation of these crystals. If crystals had not formed with the Tyr26-iodinated Y33F-CfAFP, the reciprocal tyrosine mutation and iodination yielding Tyr33-iodinated Y26F-CfAFP could have been attempted. Conceivably, this would again alter the crystal morphology and crystallization conditions of the protein owing to changes in crystal contacts, giving rise to an additional crystallization target.

The specific iodination of CfAFP resulted in a number of key events. It provided a specifically incorporated heavy atom, it led to a new crystal morphology through iodinated-tyrosine based protein–protein contacts and it allowed SAS structure determination at a home-source X-ray beam. With all of these advantages, it seems natural that specific iodination of proteins through this method can greatly assist in the structure-determination process.

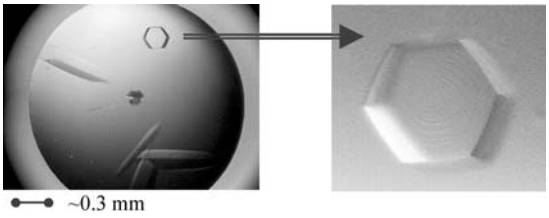

Figure 2
Crystals of iodinated Y33F-CfAFP crystallized at 318 K after initial equilibration for 24 h at room temperature. Crystallization agents included 2.0 *M* ammonium dihydrogen phosphate, 50 m*M* Tris–HCl buffer pH 7.5 and 2 m*M* cadmium chloride as an additive to the crystallization droplet.

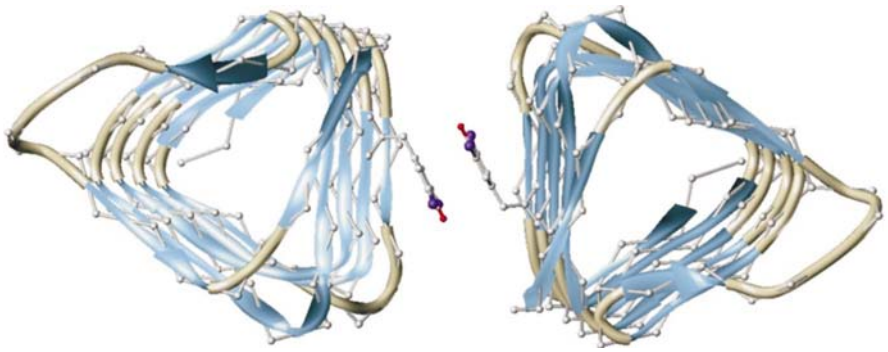

Figure 3
Iodinated tyrosine residue forms intermolecular contact between two CfAFP molecules *via* iodotyrosine stacking of equivalent tyrosine residues (PDB code 1l0s). Iodide, purple spheres; oxygen, red spheres.

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