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Crystallization and preliminary X-ray analysis of a xylanase from the psychrophile *Pseudoalteromonas haloplanktis*

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The 46 kDa xylanase from the Antarctic microorganism *Pseudoalteromonas haloplanktis* is an enzyme that efficiently catalyzes reactions at low temperatures. Here, the crystallization of both the native protein and the SeMet-substituted enzyme and data collection from both crystals using synchrotron radiation are described. The native data showed that the crystals diffract to 1.3 Å resolution and belong to space group $P2_12_12_1$, with unit-cell parameters $a = 50.87$, $b = 90.51$, $c = 97.23$ Å. SAD data collected at the peak of the selenium absorption edge proved to be sufficient to determine the heavy-atom configuration and to obtain electron density of good quality.

1. Introduction

Life on our planet has adapted to different environments. Even under extremes of pH, pressure and temperature, various microorganisms can be found. Although much research has been carried out on thermophilic and hyperthermophilic organisms, information on cold-adapted organisms is limited. In order to survive and grow in low-temperature habitats, these psychrophilic species require adaptations of their cellular content and especially of their lipids and enzymes. An enzyme needs to be flexible enough at the temperature at which it operates and, indeed, different adaptational strategies have been proposed (for recent reviews, see Zecchinon *et al.*, 2001; Russell, 2000). The tertiary structures of very few cold-adapted enzymes are known, particularly in comparison with thermophilic proteins, and as yet no general rules as to how these proteins maintain sufficient flexibility have been found. Furthermore, the structure of psychrophilic enzymes may provide clues on how to improve their stability at higher temperatures while maintaining their flexibility in a colder environment, a feature that may be useful in many biotechnological applications (Gerday *et al.*, 2000).

Here, we report on the successful crystallization and SAD phasing of the 45.98 kDa xylanase from the Antarctic bacterium *Pseudoalteromonas haloplanktis*. On the basis of its primary structure (EMBL Nucleotide Sequence Database AJ427921), the protein can be classified into glycosyl hydrolase family 8 (Coutinho & Henrissat, 1999). Curiously, this family normally comprises endoglucanases, while xylanases are usually grouped into families 10 and 11. The xylanase studied hydrolyzes xylan to principally xylotriose and xylotetraose and, in contrast to currently identified xylanases, it operates with inversion of anomeric configuration. Thus, the structure of the xylanase will provide further insights into the adaptational strategies of a macromolecule to a cold environment and will give a structural basis for the existence of xylanases within the glycosyl hydrolase family 8 enzymes.

2. Materials and methods

2.1. Crystallization

The protein was expressed and purified as described previously (Collins *et al.*, 2002). Owing to the temperature lability of the enzyme, all crystallization trials were performed at 277 K. The protein was stored at a concentration of 0.4 mg ml^{-1} in 20 m*M* MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer with 50 m*M* NaCl and 2% trehalose at a final pH of 7.5. The protein is quite stable in the stock solution, but at higher concentrations (2 mg ml^{-1} and higher) the protein crystallizes overnight without the addition of any precipitant. However, this procedure only yields showers of needles that do not dissolve even after the addition of pure water. Crystallization experiments using the hanging-drop vapour-diffusion method were performed immediately after a tenfold concentration of the protein using microconcentrators (Millipore, 10 kDa molecular-weight cutoff). Drops were prepared by mixing equal volumes of protein solution and precipitant solution. Crystal Screen I (Hampton Research) yielded 27 different crystallization conditions, with crystals of different morphologies. After testing multiple crystals and subsequent optimization, reproducible crystals with the best diffraction qualities were

obtained using 70% MPD (2-methyl-2,4-pentanediol), 0.1 *M* sodium acetate pH 5.0. These crystals appeared after one week and grew to dimensions of 300 × 300 × 100 µm (Fig. 1). The same crystallization conditions were used successfully for selenomethionine-labelled protein.

2.2. Preparation of selenomethionine-labelled protein

The xylanase expression vector (Collins *et al.*, 2002) was transformed into *Escherichia coli* B834(DE3) cells (Novagen). Transformant cells were initially precultured in a rich medium and in three successive starter cultures with increasing selenomethionine concentration. Sub-culturing and culturing were carried out in modified minimal media (MM) comprising 2× M9 (Sambrook *et al.*, 1989), 0.4% glucose, 2 m*M* $MgSO_4$, 25 µg ml^{-1} $FeSO_4.7H_2O$, all the common amino acids except methionine at 40 µg ml^{-1}, vitamins (riboflavin, niacinamide, pyridoxine monohydrochloride and thiamine) at 1 µg ml^{-1} each, 0.5 mg ml^{-1} nucleic acids and 200 µg ml^{-1} ampicillin pH 7.4.

100 µl of an overnight (310 K) Luria–Bertani (Sambrook *et al.*, 1989) culture of the transformant was inoculated into 10 ml of MM1 (40 µg ml^{-1} L-methionine). Following 12 h incubation at 310 K, 100 µl was used to inoculate 10 ml of MM2 (20 µg ml^{-1} L-methionine plus 20 µg ml^{-1} L-selenomethionine) and after 12 h at 310 K, 500 µl of this was used to inoculate 200 ml of the third starter culture (MM3) containing 40 µg ml^{-1} L-selenomethionine, and incubated at 310 K until an OD_{550} of 0.5. The cells were harvested by centrifugation at 18 000*g* for 15 min, resuspended in MM3 (40 µg ml^{-1} L-seleniomethionine) and used to inoculate 1 l of MM3. After incubation at 310 K until an OD_{550} of 1.5, the culture was induced with 1 m*M* IPTG and incubated at 291 K for 20 h. The protein was harvested and purified essentially as described previously (Collins *et al.*, 2002), with a supplement of 5 m*M* DTT and 1 m*M* EDTA in all buffers.

2.3. Data collection

A native data set was collected at beamline X13 of the EMBL Hamburg Outstation. The crystals were taken directly from the mother liquor, which already acts as a cryoprotectant, and a 1.3 Å data set was obtained at 100 K. A total of 110 images were collected with a 1° oscillation range per image. A SAD data set of the selenomethionine-labelled protein at the peak wavelength was collected at beamline BM14, ESRF, Grenoble. With a diffraction limit of 1.6 Å, a total of 100 images with a 1° oscillation range per image were obtained. Both data sets were reduced with the *HKL* package (Otwinowski & Minor, 1997).

3. Results and discussion

The protein readily crystallized under many different conditions, displaying different morphologies such as octahedra and prisms, but the majority of the crystal forms were either small or large needles. The conditions were diverse and crystals were obtained in the pH range 4.6–8.5 with very different precipitants, including different salts, polyethylene glycol (PEG), MPD, or even no precipitant at all. The only tendency observed is that the more acidic conditions (pH 4.6–6.5) tended to produce needles. Data collection on a different crystal than the one discussed indicated a $P3_1$ or $P3_2$ space group, showing that the difference in morphology may also represent a difference in the internal symmetry. The number of different crystallization conditions contrasts with the large number of crystallization trials that had to be performed for a cold-adapted amylase, the first psychrophilic enzyme for which crystals have been reported (Aghajari *et al.*, 1996). The relative ease with which the xylanase crystallizes, which was also observed for a psychrophilic alkaline protease (Villeret *et al.*, 1997), challenges the general notion that cold-adapted proteins are difficult to crystallize because of an inherent flexibility. Complete data-collection statistics are shown in Table 1. The value of the Matthews coefficient is 2.434 Å^3 Da^{-1} for a monomer in the asymmetric unit, corresponding to a solvent content of 48%, a typical value for protein crystals (Matthews, 1968).

Initially, the native data set was used in an attempt to solve the structure by molecular replacement, using the structure of a family 8 endoglucanase as a search model. These attempts failed, probably owing to the low sequence identity (23%), and a

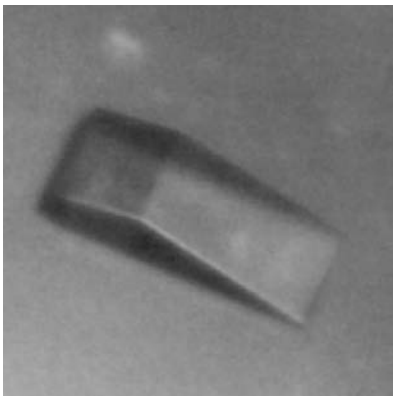

Figure 1
Crystal of the psychrophilic xylanase obtained in 70% MPD, 0.1 *M* sodium acetate pH 5.0. The longest dimension is ∼0.3 mm.

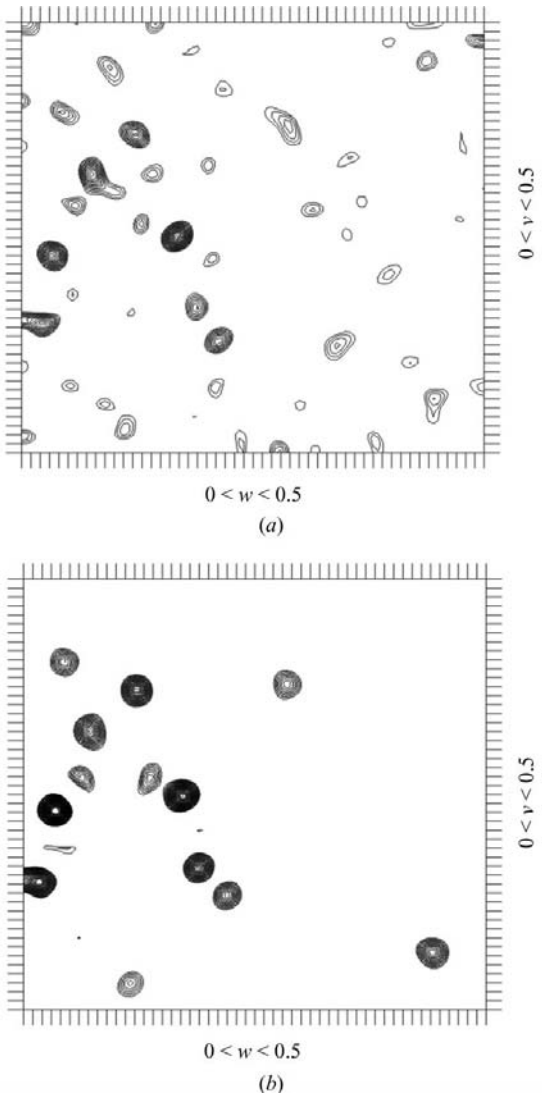

Figure 2
Harker section (u = 0.5) of the experimental anomalous Patterson map (*a*) and the theoretical Patterson map based on the predicted positions of eight selenium sites (*b*). The contour level is 2σ, with 0.5σ increments. This figure was produced with *CNS* (Brünger *et al.*, 1998).

Table 1
Statistics for data-collection and phasing.

Data collection. Values in parentheses are for the highest resolution shell of each data set.

	Native	Seleno-methionine
Wavelength (Å)	0.8015	0.9789
Detector type	MAR CCD	MAR CCD
Resolution (Å)	20.0–1.3 (1.35–1.30)	20.0–1.60 (1.66–1.60)
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 50.87, b = 90.51, c = 97.23	a = 51.36, b = 91.10, c = 97.69
Total No. of reflections	736429	381108
No. of unique reflections	111031	61029
Completeness (%)	99.2 (99.3)	95.8 (93.7)
R_{sym}†	3.9 (32.6)	2.6 (4.9)
$\langle I/\sigma(I)\rangle$	29.6 (3.6)	31.9 (17.2)
Mosaicity (°)	0.213	0.154

Phasing statistics for ten selenium sites.

R_{cullis}	0.32
Phasing power centrics/acentrics	4.36/4.24
FOM before density modification	0.52
FOM after density modification	0.93

† $R_{sym} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i (h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the average value over multiple measurements.

MAD experiment was subsequently planned. In order to determine the peak and inflection wavelength, a fluorescence scan was recorded on an SeMet-substituted crystal. All methionines had been successfully replaced, as indicated by mass spectrometry, which showed a 100% incorporation of selenomethionine (results not shown). A total of ten methionines are present per 46 kDa in the asymmetric unit. The data collected at the peak wavelength proved to be sufficient to produce an interpretable anomalous Patterson map and eight of the sites could readily be found using the program *CNS* (Brünger *et al.*, 1998) (Fig. 2). In a 100-trial run, the solution with a correlation factor of 53% was found 20 times and clearly stands above the next best solution (49%), as well as above the mean correlation factor of all wrong solutions (35%). Further refinement of the substructure, phasing, completion of the heavy-atom substructure and further density modification were also carried out using *CNS*. After choosing the proper hand, an interpretable electron-density map was obtained in which the main chain and some side chains were clearly visible. Tracing of the whole main chain and refinement and a full description of the phasing are under way.

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