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Crystallization and X-ray diffraction measurements of a thermophilic archaeal recombinant amidase from *Sulfolobus solfataricus* MT4

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Recombinant amidase is a 55.8 kDa enzyme from the thermophilic archaeon *Sulfolobus solfataricus* MT4 that catalyses the hydrolysis of aliphatic amides of 2–6 C atoms as well as many aromatic amides. Single crystals of purified amidase were obtained by the hanging-drop method at 294 K. Diffraction data for the native protein (2.55 Å resolution) and a putative derivative (2.20 Å) have been collected at low temperature using synchrotron radiation. The crystals belong to the rhombohedral space group *R*3. Structure determination by multiple isomorphous replacement is in progress. It is expected that structural information from this signatured thermostable amidase will increase our knowledge of the molecular mechanisms employed to maintain high-temperature stability in thermophilic proteins.

1. Introduction

Amidases are widely distributed in both prokaryotic and eukaryotic organisms. They act on C—N bonds other than peptide bonds (E.C. 3.5) and are of particular interest owing to their potential industrial applications (Nagasawa & Yamada, 1989; Kobayashi *et al.*, 1992). Amidases catalyze the hydrolysis of various endogenous and exogenous aliphatic and aromatic amides by transferring an acyl group to water, with the production of free acids and ammonia.

Amidases may be grouped into two classes: the first class contains a characteristic GGSS-(S/G)GS signature found in more than 45 proteins (Mayaux *et al.*, 1991), while the second class, the so-called aliphatic amidases, lack this signature. The amidases belonging to the first class show a remarkable variance in their individual substrate specificities, can hydrolyze aliphatic and/or aromatic substrates, share many common features in their primary structures and are usually homodimers. The prototype of the second class of amidases was isolated from *Pseudomonas aeruginosa* (Ambler *et al.*, 1987). It is a hexamer and hydrolyzes only small aliphatic substrates such as acetamide and propionamide.

Recently, we have cloned, sequenced and overexpressed in *Escherichia coli* the amidase gene from the hyperthermophilic archaeon *S. solfataricus* (strain MT4). The recombinant protein was obtained as a fusion protein with a His tag (Scotto d'Abusco *et al.*, 2001). The protein is the first characterized archaeal signatured amidase. It has a molecular weight of 55 784 Da. The enzyme, which can distinguish enantiomers, is active on aliphatic amides of 2–6 C atoms as well as on many aromatic amides. It is active in the pH range 4–9 and in the temperature range 333–368 K. It could be used in industrial processes for poorly water-soluble substrates as it retains its activity in a 50% mixture of methanol, ethanol and propanol. The lack of any three-dimensional structure of a signatured amidase and the possibility of increasing our knowledge of the molecular mechanisms employed to maintain high-temperature stability in thermophilic proteins (Scandurra *et al.*, 1998) prompted us to initiate the crystal structure determination of a recombinant amidase from *S. solfataricus* MT4 without a His tag. In the current work, we report the purification, crystallization and the preliminary diffraction data for the native protein at 2.55 Å and for a putative derivative at 2.20 Å resolution.

2. Materials and methods

2.1. Protein preparation and crystallization

The expression of the amidase was performed by amplifying the amidase gene by PCR and inserting the PCR product into pET3d expression vector. This expression vector takes advantage of the T7 bacteriophage gene 10, which promotes high-level transcription and translation. The clone λ5C1, described in Scotto d'Abusco *et al.* (2001), was used as a template. The PCR reaction was performed with primers AMSC3 (5′-GTCCA-TGGGAATTAAGTTACCCA-3′) and AMSC2 (5′-GAGAGGATCCTTATTTTTTGATTCTC-3′) under the following conditions: 367 K for

1 min, 329 K for 1 min and 345 K for 2 min, repeated 30 times. The PCR product was digested with *Nco*I and *Bam*HI, inserted into pET3d (Invitrogen) and digested again with the same pair of enzymes. After confirmation by DNA sequencing, the construct obtained, pASCe, was transformed into *E. coli* BL21(DE3) plys S strain for protein expression. Cells were grown at 310 K in LB ampicillin (100 µg ml^{-1}) adding 0.4 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) when the absorbance of the culture was 1 at 600 nm. Cells were harvested by centrifugation and disrupted by sonication. The recombinant enzyme found in the insoluble fraction was extracted three times with 20 m*M* sodium phosphate buffer pH 7.8, 500 m*M* NaCl, 0.25%(*w*/*v*) Tween 20. Portions of 5 mg of protein were applied onto a HiLoad 16/60 Superdex 200 column (Pharmacia) and eluted with 20 m*M* sodium phosphate buffer pH 7.0, 150 m*M* NaCl at 1 ml min^{-1}. The fraction of interest was concentrated with an Amicon pressurized device equipped with a PM 30 membrane. Gel electrophoresis, performed under either native or denaturing conditions, demonstrated the purity of the protein (>98%).

Crystallization experiments were first performed using a fusion protein with a His tag. Despite extensive variation of the crystallization conditions, duplicated at 294 and 310 K, we were unable to obtain crystals. Removal of the His tag resulted in small crystals: a cloudy precipitate forms within a few hours and crystals grow within two weeks. They are well formed trigonal prisms but are few in number, usually no more than one or two crystals per drop, and their dimensions do not exceed 40 µm. The protein solution for crystallization (11 mg ml^{-1}) consisted of 20 m*M* sodium phosphate buffer pH 7.0, 150 m*M* NaCl, 100 µ*M* EDTA, 200 µ*M* DTT. Crystals were obtained by mixing equal volumes (2 µl) of protein solution and reservoir solution on a siliconized cover slip. The drops were vapour-equilibrated against 1.0 ml of the reservoir solution at 294 K using the hanging-drop vapour-diffusion technique (McPherson, 1990). Crystal formation was observed in 57–64% ammonium sulfate in the pH range 7.0–9.5, the size and shape of the crystals being independent of the crystallization conditions. Trials to increase the size of the crystals (varying the protein concentration and the crystallization geometry, removal of the precipitated protein and addition of new protein to the drop, varying the precipitants, detergents, glycerol, salts and temperature) were unsuccessful, but larger drops (7 µl) and macroseeding did yield slightly larger crystals. The crystals used for data collection at the synchrotron were roughly 0.05 × 0.04 × 0.02 mm in size; the reservoir solution consisted of 62% saturated ammonium sulfate, 100 m*M* HEPES pH 7.5, 1 m*M* NaN_3.

2.2. Cryofreezing and data collection

Measurements were made at 100 K (Rodgers, 1994) using 25% glycerol in the mother liquor as cryoprotectant. Data were collected at the ID14-1 beamline station, ESRF (Grenoble, France) using a MAR CCD detector. A native and a putative mercury derivative were measured at a wavelength of 0.934 Å, which is close to the absorption edge of mercury. For the native crystal we used an oscillation of 0.7° over a range of 90° and for the derivative an oscillation of 1.0° over 150°. Interestingly, the derivative diffracted better than the native. The relevant statistics are summarized in Table 1. The derivative was prepared by soaking a crystal in a solution of mother liquor containing 5 m*M* *p*-hydroxymercuribenzoate for 16 h. The data were indexed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). Subsequent calculations were performed using the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The crystals belong to the rhombohedral space group *R*3, with unit-cell parameters $a = b = 108.35$, $c = 154.51$ Å for the native and $a = b = 108.55$, $c = 153.83$ Å for the derivative. The native data were collected to a resolution of 2.2 Å but were processed to 2.55 Å because of an intensity decrease owing to radiation damage. The resolution of the derivative is 2.20 Å, which is quite strong given the small crystal size. The calculated V_M value (Matthews, 1968) assuming one molecule in the asymmetric unit is 3.12 Å^3 Da^{-1}, which corresponds to a solvent content of 61%.

The analysis of the derivative data set indicates that it could indeed be used to solve the structure in conjunction with additional data sets; therefore, our aim is to determine the three-dimensional structure of the amidase either through the use of the multiple isomorphous replacement method or by making use of the mercury as an anomalous scatterer in the multiwavelength anomalous dispersion method (MAD). This structure determination will add to our knowledge of thermophilic enzymes in addition to being the first structure of a signatured amidase.

Table 1
Data-collection parameters.

(*a*) Statistical data-collection parameters for the native amidase crystal as a function of resolution.

Resolution (Å)	$I > 3\sigma(I)$ (%)	χ^2†	R_{merge}‡	Completeness (%)
20.0–4.35	88.6	0.937	0.052	96.0
4.35–3.46	79.7	0.792	0.078	96.8
3.46–3.02	62.6	0.664	0.138	97.7
3.02–2.75	42.7	0.486	0.227	98.3
2.75–2.55	28.5	0.428	0.382	98.3
Overall	60.3	0.654	0.100	97.4

(*b*) Statistical data-collection parameters for the derivative amidase crystal as a function of resolution.

Resolution (Å)	$I > 3\sigma(I)$ (%)	χ^2†	R_{merge}‡	Completeness (%)
20.0–3.76	93.4	0.901	0.052	99.8
3.76–2.98	81.1	0.585	0.079	99.9
2.98–2.61	59.5	0.395	0.150	100.0
2.61–2.37	39.5	0.351	0.258	100.0
2.37–2.20	28.7	0.337	0.365	99.9
Overall	60.4	0.513	0.091	99.9

† $\chi^2 = \sum_h \sum_i (I_{hi} - \langle I_h \rangle)^2 / [\sigma_h^2 N/(N-1)]$, where N is the number of observations. ‡ $R_{merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$, where I_{hi} is the ith observation of the reflection h and $\langle I_h \rangle$ is the mean intensity of the hth reflection.

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