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Expression, purification and preliminary crystallographic studies of a hyperthermophilic esterase from *Archaeoglobus fulgidus*

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An esterase from the hyperthermophilic archeon *Archaeoglobus fulgidus* has been expressed, purified and crystallized in a form suitable for structure analysis. The enzyme has a molecular mass of 35 467 Da and shows sequence similarity to other esterases known to possess the α/β hydrolase fold. The crystals diffract to 2.8 Å and belong to space group $I222$ or $I2_12_12_1$, with unit-cell parameters $a = 155.6$, $b = 155.0$, $c = 162.4$ Å.

1. Introduction

Esterases are the most common enzymes found in living systems. The α/β hydrolase fold enzymes probably represent the most diverse group of esterases yet characterized; they include lipases and choline esterases found in higher organisms as well as numerous bacterial enzymes used in the breakdown of toxic materials (Ollis *et al.*, 1992). Although there is little sequence similarity between all the α/β hydrolase fold enzymes, there is clear structural similarity in the catalytic core of the enzymes. We wish to further characterize the divergent nature of the α/β hydrolase fold enzymes and have identified an esterase in a hyperthermophilic archeon *A. fulgidus* whose sequence can be aligned with other α/β hydrolase fold enzymes. The level of absolute sequence similarity to most other members of the group is low. However, it does show 31% sequence identity with an aryl esterase (AreA, accession no. 4929533) from *Acinetobacter* (Jones *et al.*, 1999). We have isolated the esterase gene, expressed it at high level in *Escherichia coli*, purified it to homogeneity and obtained crystals for structure analysis.

2. Materials and methods

2.1. Expression

The DNA sequence encoding an esterase (estA, accession No. 2648837) was amplified using the polymerase chain reaction (PCR) with genomic DNA from *A. fulgidus* VC-16. The upper primer had the sequence 5′-GCC CGG GAT CCA CAT ATG CTT GAT ATG CCA ATC GAC-3′, while the lower primer had the sequence 5′-CCG TCT CGA GAA TTC TTA CTA GTC GAA CAC AAG AAG AGC-3′. Standard PCR conditions were used with *Pfu* DNA polymerase (Stratagene). The amplified fragment was purified with a PCR purification kit from QIAGEN and was digested with restriction enzymes *Nde*I and *Eco*RI. The resulting DNA fragment (941 bp) was cloned into a T7 expression vector pET 22b cut with the same enzymes to yield the plasmid pJWL1005.

2.2. Purification and characterization

The *A. fulgidus* esterase was overexpressed in *E. coli* strain BL21(DE3) bearing plasmid pJWL1005. Four 1 l cultures of Luria broth containing 100 µg ml^{-1} of ampicillin were inoculated with 40 ml of overnight starter cultures and grown at 310 K. When the OD_{595} reached 0.3–0.4, the cultures were induced by adding IPTG to a final concentration of 0.5 m*M*. Growth continued for a further 2 h. The cells were harvested by centrifugation at 8300*g* for 10 min. The pellets were resuspended in buffer *A* (20 m*M* HEPES pH 7.0, 1 m*M* EDTA) and lysed in a French pressure cell. The lysate was heated at 348 K for 10 min. Cell debris and precipitated proteins were removed by centrifugation at 39 000*g* for 30 min. The proteins in the supernatant were loaded onto a DEAE column previously equilibrated in buffer *A*. After washing the column with 10% buffer *B* (buffer *A* containing 1 *M* NaCl), the *A. fulgidus* esterase was eluted with a linear gradient of 10–500 m*M* NaCl. The esterase eluted between 180–330 m*M* NaCl and gave a prominent peak on the chromatogram. Sodium dodecyl sulfate (1%) polyacrylamide gel (20%) electrophoresis (SDS–PAGE) confirmed that this peak contained the partially purified protein. The peak fractions were pooled and loaded onto a Sephadex G75 column that had been previously equilibrated with buffer *A*. The purified protein was concentrated using an Amicon Centricon 30 to 9.8 mg ml^{-1} for crystallization trials. Protein concentration was determined by a UV spectrophotometer at OD_{280}.

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The activity of the enzyme was measured spectrophotometrically. Activity against *para*-nitrophenol (pNp) acetate, pNp propionate and pNp valerate was measured by monitoring the formation of an absorption at 410 nm. Activity against benzyl esters was detected using a coupled assay in which the acid product of ester hydrolysis was acted upon by an alcohol dehydrogenase that produced NADH. The extent of hydrolysis could be determined by a change in absorption at 340 nm as described by Jones *et al.* (1999).

2.3. Crystallization

Hampton Crystal Screen I, which is based on the sparse-matrix sampling of Jancarik & Kim (1991), was used to test 48 crystallization conditions. The screen was carried out at 277 and 291 K, but the better crystals were obtained at the higher temperature and all subsequent work was performed at this temperature. Grid screens at 291 K were preformed with Linbro multiwell tissue-culture plates in which 4 µl of protein solution was added to 4 µl of reservoir solution in a hanging drop suspended over a 500 µl reservoir. Crystals suitable for X-ray diffraction studies were grown using a reservoir solution that contained 2-propanol, PEG 4000 and trisodium citrate dihydrate. Optimal conditions for crystallization were achieved with reservoir solutions consisting of 10–20% 2-propanol, 20% PEG 4000 and 0.1 *M* trisodium citrate dihydrate pH 5.6–6.0. Crystals grown under these conditions could be reproducibly grown to dimensions of 0.75 × 0.75 × 0.5 mm.

2.4. Data collection and analysis

A crystal was transferred to a cryobuffer solution containing 15% 2-methyl-2,4-pentanediol (MPD), 20% PEG 4000 and 0.1 *M* trisodium citrate dihydrate pH 6.0. The crystal was flash-cooled to 100 K in a nitrogen stream produced by a modified MSC (Molecular Structure Corporation) cooling system as described by Carr *et al.* (1996). X-ray data were collected with an R-AXIS II detector mounted on a Rigaku generator with a graphite monochromator. The generator produced Cu $K\alpha$ radiation at a power of 5 kW (59 kV, 84 mA). The data were processed using the program *HKL* version 1.90 (Minor, 1993; Otwinowski, 1993). The data set comprised of 152 825 observations of 42 426 unique reflections (88.1% complete to 2.8 Å), with $R_{\rm sym}$ = 9.7% and $\langle I/\sigma(I)\rangle$ = 12.2. Post-refined values of the unit-cell parameters were a = 155.6, b = 155.0, c = 162.4 Å. A high value for $R_{\rm sym}$ (39.2%) was obtained when the data were processed using a tetragonal cell. This eliminated the possibility that the crystals were tetragonal. The diffraction pattern obtained from 1° oscillation of the crystal at low temperature is shown in Fig. 1. The value of the Matthews coefficent (Matthews, 1968) is 3.5 Å^3 Da^{-1} for four monomers in the asymmetric unit.

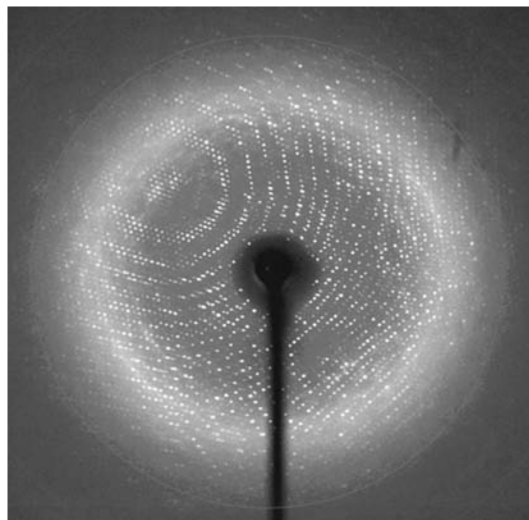

Figure 1
X-ray diffraction image from a 1° oscillation of the crystal. The line indicates the 2.8 Å diffraction limit.

3. Conclusions

The protein was expressed at a high level as indicated by the fact that it was the dominant band on a Coomassie-stained SDS gel of the crude lysate. The purification produced 34 mg of protein, which was better than 95% pure as judged by inspection of a gel. Activity was detected with pNp acetate, pNp propionate and pNp valerate. Activity was also detected with the benzyl esters of propionic acid and benzoic acid. Activity was detected against a range of compounds, indicating that the esterase had a broad range of substrates similar to that of the aryl esterase identified by Jones *et al.* (1999). It was also found that the activity of the enzyme increased with temperature up to 338 K.

The structure determination is in progress, with some success in the search for heavy-atom derivatives.

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