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Crystallization and preliminary X-ray diffraction studies of Aes acetyl-esterase from *Escherichia coli*

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The acetyl-esterase Aes from *Escherichia coli*, which belongs to the HSL group of the esterase/lipase superfamily, has been crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 8000 as a precipitant and magnesium chloride as an additive. Crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 110.0$, $b = 190.6$, $c = 218.6$ Å. A complete data set has been collected to 2.5 Å resolution at the Elettra synchrotron source, Trieste using a single frozen crystal. Packing density considerations agree with 10–16 monomers in the asymmetric unit, with a corresponding solvent content of 61–38%.

1. Introduction

The *Escherichia coli aes* (or *ybac*) gene encodes the 36 kDa cytoplasmic protein Aes (Peist *et al.*, 1997), a carboxylesterase belonging to the hormone-sensitive lipase (HSL) group of the esterase/lipase superfamily (Kanaya *et al.*, 1998). Although the physiological role of this enzyme is still obscure, it has recently received greater attention owing to the discovery that Aes is involved in the regulation of the maltose regulon in *E. coli* (reviewed in Boos & Shuman, 1998). The *E. coli* maltose system consists of ten genes encoding proteins for the uptake and metabolism of maltodextrin and maltose (Boos & Shuman, 1998). MalT is the transcriptional activator of the maltose regulon and is the prototype of a new family of transcription factors (De Schrijver & De Mot, 1999; Valdez *et al.*, 1999); it integrates several signals through its three N-terminal domains (Danot, 2001). To activate *mal* gene transcription, MalT acts together with the cyclic AMP–catabolite protein complex, the inducer maltotriose and ATP (Richet & Raibaud, 1989). Several regulatory circuits modulate the activity of MalT, *e.g.* MalT expression is regulated by Mlc, a glucose-inducible repressor (Decker *et al.*, 1998). In addition, MalT is negatively controlled by at least three proteins: MalY, MalK and Aes. MalY is an enzyme with cystathionine β-lyase activity (Reidl & Boos, 1991; Zdich *et al.*, 1995) which interacts directly with the N-terminus of MalT (Schlegel *et al.*, 2002; Schreiber *et al.*, 2000), seemingly through a patch made up of a hydrophobic core surrounded by highly polar residues (Clausen *et al.*, 2000). MalK, the ATP-hydrolyzing subunit of the maltose-transport system (Kuhnau *et al.*, 1991), has been reported to interact directly with MalT (Panagiatodis *et al.*, 1998), probably through several residues located in its C-terminal domain (Böhm *et al.*, 2002). With regard to the Aes protein, it has been demonstrated that Aes controls MalT activity through a direct protein–protein interaction that counteracts the binding of the MalT effector maltotriose. It was postulated that the Aes-binding site is mostly located in the DT1 domain of MalT, which also contains the ATP-binding site (Joly *et al.*, 2002). Accordingly, ATP and ADP differently affect the competition between Aes and the inducer (maltotriose) as regards binding to MalT. The Aes and MalY sites of interaction on MalT are probably superimposed, while MalK would appear to interact at a different location (Schlegel *et al.*, 2002). Thus, it is possible that MalY and Aes share similar structural determinants, but these motifs cannot be easily predicted simply on the basis of sequences analysis (Clausen *et al.*, 2000).

It has been reported that the basal level of *aes* expression is very low (Peist *et al.*, 1997). However, the introduction of a plasmid harbouring the *aes* gene was required to allow *E. coli* growth on minimal medium supplemented with triacetylglycerol (triacetin) as the sole carbon source. Moreover, the existence of a repressor of the *aes* gene has also been postulated (Peist *et al.*, 1997), thus suggesting the importance of this enzyme under particular growth conditions.

We have been working for several years on the structure–function relationships of thermostable carboxylesterases from the HSL family (Manco *et al.*, 1998, 2000). Recently, we have reported the three-dimensional structures of EST2 from *Alicyclobacillus acidocaldarius*, a thermophilic eubacterium (De Simone *et al.*, 1999, 2000), and the homologous enzyme AFEST from the hyperthermophilic archaeon *Archaeoglobus fulgidus* (De Simone *et al.*,

2001). We have also reported structural and thermodynamic studies on both enzymes (D'Auria *et al.*, 2000; Manco *et al.*, 2001; Del Vecchio *et al.*, 2002). In order to perform a comparative analysis with a mesophilic counterpart, we cloned the *aes gene* from *E. coli* genomic DNA, overexpressed the protein in *E. coli* and purified it. Quite surprisingly, we isolated an 87 kDa complex which had never been previously described involving the Aes enzyme and the *E. coli* α-galactosidase, an enzyme belonging to the family 4 of the glycosylhydrolases. In this complex, Aes was active whereas the α-galactosidase was inactive. A dimeric version of Aes was also detected, suggesting a high propensity of this enzyme towards protein–protein interactions (Mandrich *et al.*, 2002). In order to provide new insights into the structural determinants of the Aes protein-interaction properties, we started a project for solution of the three-dimensional structure of this enzyme.

2. Materials and methods

2.1. Protein expression and purification

Overexpression and purification of protein was essentially performed as described previously (Mandrich *et al.*, 2002). Briefly, *E. coli* BL21 (DE3) cells were transformed with the pT7-SCII-aes construct and cultured in 5 l of Luria–Bertani (LB) medium supplemented with 100 µg ml^{-1} of ampicillin. Cells at a density corresponding to an optical density value of 1 at 600 nm were treated with 0.5 m*M* IPTG for 4 h. Thereafter, cells (40 g wet weight) were harvested by centrifugation (3000*g*, 277 K, 10 min), washed with 25 m*M* Tris–HCl buffer pH 8.5, 2.5 m*M* $MgCl_2$, 0.5 m*M* EDTA (buffer *A*) and stored at 253 K. Finally, when required, cells were thawed and redissolved in 100 ml buffer *A*. Disruption was achieved with a French pressure cell and cellular debris was removed by centrifugation (80 000*g*, 277 K, 20 min).

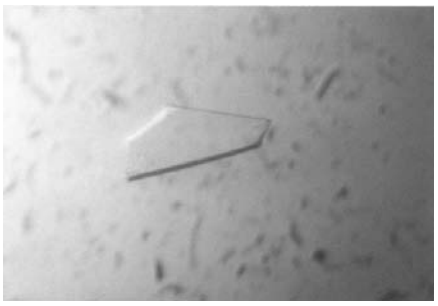

Figure 1
Crystals of Aes. Crystal dimensions are 0.05 × 0.2 × 0.3 mm.

The crude extract was treated with 10%(*w*/*v*) ammonium sulfate and centrifuged (17 000*g*, 277 K, 20 min). The supernatant was brought to 40%(*w*/*v*) ammonium sulfate and centrifuged again (17 000*g*, 277 K, 20 min). The pellet was redissolved in 100 ml buffer *A* and loaded onto a Q-Sepharose Fast Flow column (20 × 2.6 cm, Amersham Pharmacia, Uppsala, Sweden) equilibrated in buffer *A*. After two volumes of washing, a linear gradient of NaCl (from 0 to 1 *M*) was applied. Active fractions were pooled, brought to 10%(*w*/*v*) ammonium sulfate and loaded onto a HiLoad 16/10 Phenyl Sepharose column (Amersham Pharmacia, Uppsala, Sweden) equilibrated in buffer *A* containing 10% ammonium sulfate. Proteins were eluted with a decreasing gradient (10–0%) of ammonium sulfate in buffer *A*. Esterase activity was eluted and active fractions were pooled, dialyzed against buffer *A* and fractionated on a Mono Q HR 5/5 column eluted with a linear gradient of NaCl (0–1 *M*). Most of the applied activity was eluted at 300 m*M* NaCl. The most active fractions were pooled, dialyzed against buffer *A* and stored at 277 K until use. Preparation of a selenomethionine derivative of Aes was performed as previously described for EST2 from *Alicylobacillus acidocaldarius* (De Simone *et al.*, 2000); purification was similar to that of wild-type Aes.

2.2. Crystallization and X-ray analysis

The purified native protein (4.6 mg ml^{-1}) was crystallized at 293 K. Crystallization conditions were screened according to the sparse-matrix method (Jancarik & Kim, 1991) using commercially available buffers (Hampton Research, Laguna Hills, CA, USA) and the hanging-drop vapour-diffusion technique. PEG 8000 was found to be the best crystallization precipitant. Other parameters such as buffer composition, pH and temperature were varied in order to improve the crystal quality. The best crystallization condition consisted of mixing 1 µl of protein solution with 1 µl of reservoir solution containing 100 m*M* sodium cacodylate pH 5.0, 200 m*M* magnesium acetate and 8%(*w*/*v*) PEG 8000. Under these conditions, crystals could be reproducibly grown to dimensions of 0.05 × 0.2 × 0.3 mm in one week (Fig. 1).

For data collection, crystals were (i) briefly washed in solutions of 100 m*M* sodium cacodylate pH 5.0, 200 m*M* magnesium acetate, 8%(*w*/*v*) PEG 8000 and 25%(*v*/*v*) glycerol, (ii) retrieved with a 0.3 mm nylon loop and (iii) flash-frozen in the nitrogen-gas stream from an Oxford Cryostream cooler operated at a temperature of 100 K.

Table 1
Crystal and data-collection parameters.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	110.0
b (Å)	190.6
c (Å)	218.6
Resolution limits (Å)	20.0–2.50
Temperature (K)	100
Total reflections	2200790
Unique reflections	160567
Completeness (%)	84.6 (55.5)
R_{sym}† (%)	11.0 (37.7)
Mean $I/\sigma(I)$	9.05 (2.17)

† $R_{sym} = \sum |I_i - \langle I \rangle| / \sum I_i$ over all reflections.

X-ray diffraction data were collected on a MAR CCD detector at the Elettra synchrotron source, Trieste and were processed using the *DENZO* and *SCALEPACK* crystallographic data-reduction package (Otwinowski & Minor, 1997).

3. Results

Large well formed crystals of Aes (Fig. 1) were obtained as described above. Upon diffraction using synchrotron radiation, data were collected to a resolution of 2.5 Å. Table 1 reports crystal data and data-collection statistics. Packing-density considerations (Matthews, 1968) for a monomer mass of 35 963 Da suggested 10–16 monomers in the asymmetric unit (V_M = 3.18–1.99 Å^3 Da^{-1}, corresponding to a solvent-content range of 61–38%).

A self-rotation function calculation performed with the *GRLF* program (Tong & Rossmann, 1990) using different integration radii and resolution ranges did not give conclusive information about the asymmetric unit content. To elucidate the answer to this question, a complete structure determination will be required.

The low sequence identity with proteins of known three-dimensional structure and the availability of the SeMet-containing enzyme suggested multiple-wavelength anomalous diffraction (MAD; Hendrickson & Ogata, 1997) as the method of choice for Aes structure analysis. Introduction of SeMet in the protein was performed as previously reported (De Simone *et al.*, 2000), whilst purification of SeMet-substituted Aes was performed as described for the native protein (see §2). From the last chromatographic step, one prominent active peak was obtained, presenting a molecular-weight

increase of 476 Da with respect to the native Aes as judged by mass-spectrometry analysis. This difference was associated with the introduction of ten S→Se atom substitutions (theoretical weight difference: 477 Da), corresponding to complete methionine-residue replacement. At the moment, the seleniomethionine derivative is being crystallized.

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