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Crystallization and preliminary X-ray analysis of the *ytxM* gene product from *Bacillus subtilis*

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The *ytxM* gene product from *Bacillus subtilis* has been cloned, expressed in *Escherichia coli*, purified and crystallized by the hanging-drop vapour-diffusion method using PEG 3350 as the precipitant. Multiple-wavelength anomalous dispersive X-ray data have been collected to 2.0 Å resolution on a single selenomethionine-incorporated crystal. This crystal belongs to the primitive orthorhombic system, with approximate unit-cell parameters a = 44.3, b = 90.9, c = 136.1 Å, $\alpha = \beta = \gamma = 90^\circ$ and two monomers in the asymmetric unit.

1. Introduction

The elucidation of the genomic sequences of many prokaryotes has highlighted the vast gaps in our understanding of the identity and roles of many gene products. For instance, 40% of the 4200 genes in *Bacillus subtilis* at the time of release of the genomic sequence were of unknown function (Kunst *et al.*, 1997). One route towards identifying the biochemical and biological function of these proteins is through the determination of their structures. *ytxM* was originally identified as one of a number of putative essential genes of unknown function following a functional genomic analysis of *B. subtilis* and encodes a 274-amino-acid, 30.5 kDa molecular-weight protein (herein after referred to as YtxM). Primary sequence comparisons using the program *BLAST* (Altschul *et al.*, 1997) have identified significant levels of sequence similarity between YtxM and five proteins of known three-dimensional structure: *Streptomyces aureofaciens* bromoperoxidase A2 (BP; Hecht *et al.*, 1994; Hofmann *et al.*, 1998), *S. lividans* chloroperoxidase L (CP; Hofmann *et al.*, 1998), murine soluble epoxide hydrolase (EH; Argiriadi *et al.*, 2000), *Serratia marcescens* prolyl amino peptidase (PAP; Yoshimoto *et al.*, 1999) and *Xanthomonas campestris* pv. *citri* proline iminopeptidase (PIP; Medrano *et al.*, 1998), with sequence identities ranging between 13.9% (PAP) and 22.9% (BP) (Fig. 1). These proteins belong to the α/β-hydrolase superfamily and inspection of the aligned sequences has revealed that the catalytic triad of an aspartate, histidine and serine identified in four of these proteins (CP, BP, PAP and PIP) is also conserved in YtxM (corresponding to

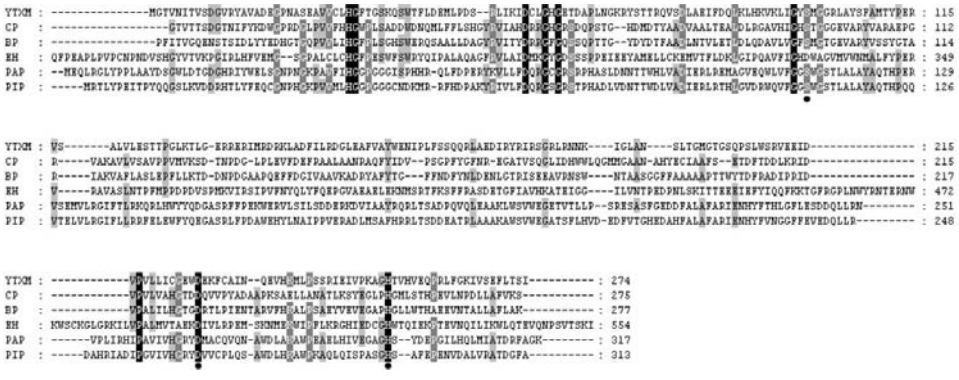

Figure 1
Multiple sequence alignment of the sequence of YtxM with members of the α/β-hydrolase superfamily: *S. lividans* chloroperoxidase L (CP), *S. aureofaciens* bromoperoxidase A2 (BP), murine soluble epoxide hydrolase (EH), *S. marcescens* prolyl amino peptidase (PAP) and *X. campestris* proline iminopeptidase (PIP). Fully conserved residues are highlighted in reverse type and highly conserved residues are shaded according to the criterion of *BLO-SUM*62 (Henikoff & Henikoff, 1992). Residues highlighted by a black dot below the aligned sequences represent the catalytic triad of aspartate, histidine and serine previously identified within this enzyme family. The multiple sequence alignment was prepared using *CLUSTAL* (Higgins *et al.*, 1992).

residues Ser99, Asp226 and His253). In order to assist in the identification of the biological function and mode of action of YtxM, we have initiated a full structural study.

2. Materials and methods

The *ytxM* gene from *B. subtilis* 168 was PCR amplified from genomic DNA using primers carrying the *Nde*I and *Bgl*II restriction sites. The PCR product (822 bp) was purified (Qiagen) and cloned into the pTB361 vector under the control of a T7 promoter to produce a new construct, pTB361-*ytxM*, that was used to transform competent cells of *E. coli* DH5α. Positive clones were identified by both PCR screens and restriction-enzyme digestion. DNA sequencing was used to validate the incorporated gene and the plasmid was subsequently used to transform competent cells of the overexpression strain *E. coli* Tuner (DE3) pLacI (Novagen). A single colony was cultured in LB medium containing tetracycline (15 µg ml^{-1}) with vigorous aeration at 310 K until the turbidity reached OD_{600} = 0.7. Cells were induced with IPTG (0.5m*M* final concentration) and were subsequently grown at 298 K for a further 4 h.

For production of the selenomethionine-incorporated protein, the pTB361-*ytxM* construct was used to transform competent cells of the overexpression strain *E. coli* B834 (DE3) Met^{-}. A single colony was cultured overnight at 310 K in 100 ml LB medium containing tetracycline (15 µg ml^{-1}). The cells were harvested by centrifugation at 2000*g* and resuspended in 50 ml minimal medium. 5 ml of this resuspension was used to inoculate 500 ml of minimal medium in a 2 l flask supplemented with all of the natural L-amino acids (40 mg l^{-1}) but with methionine substituted by selenomethionine. The cells were subsequently grown at 310 K until the turbidity reached OD_{600} = 0.8, then induced as previously described and harvested in a Beckman J8-ME refrigerated centrifuge (277 K) at 5000*g*. The cell pellets were kept frozen at 253 K.

For purification, the wet weight of cells obtained from a 3 l culture was defrosted, suspended in buffer *A* (40 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA) and disrupted by ultrasonication for 3 × 20 s at 16 µm amplitude. Cell debris was removed by centrifugation at 45 000*g* for 15 min using a JA-20 rotor in a Beckman refrigerated centrifuge (277 K). In total, the supernatant fraction contained approximately 400 mg of protein that was applied to a 30 ml DEAE-Sepharose Fast Flow column (Pharmacia) equilibrated in buffer *A*. The proteins were eluted using a 300 ml linear gradient of NaCl from 0 to 0.25 *M* in buffer *A*. Fractions were collected and the protein concentration measured by the method of Bradford (1976) using the Bio-Rad dye reagent and the purity analysed by SDS–PAGE (Nu Page 4–12% Bis–Tris gel, Novex). The fractions containing the highest YtxM content were combined to give a total of 45 mg of protein and were concentrated on a Viva-Spin concentrator (Viva Science; 10 kDa molecular-weight cutoff) and applied onto a 1.6 × 60 cm Hi-Load Superdex-200 gel-filtration column (Pharmacia) equilibrated with buffer *B* (0.1 *M* NaCl, 20 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA). YtxM was eluted from the column with buffer *B* and the peak fractions combined and concentrated on a Viva-Spin concentrator to a final protein concentration of approximately 23 mg ml^{-1}.

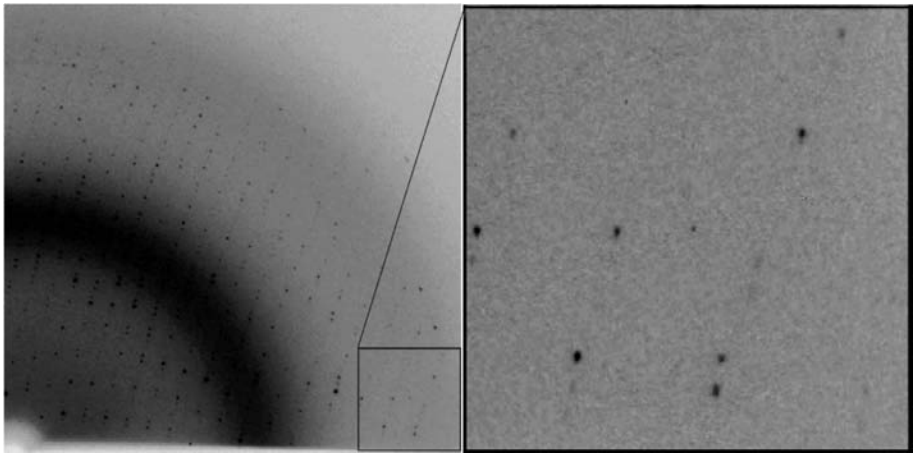

Figure 2
A representative quadrant of a diffraction image from the frozen (100 K) YtxM selenomethionine-incorporated crystal.

To purify the selenomethionine-incorporated protein, an additional hydrophobic chromatography step was performed using a 20 ml phenyl Toyopearl 650S column (Toyo-Soda). The protein was loaded onto the column in the presence of 2 *M* ammonium sulfate and eluted using a reverse ammonium sulfate concentration gradient from 1.8 to 0 *M* in buffer *A*.

3. Results and discussion

The level of overexpression of selenomethionine-incorporated YtxM was estimated to be about 5% of the total soluble cell protein. The purity of YtxM by SDS–PAGE (4–12% Bis–Tris Nu-PAGE gel, Novex) was estimated to be between 75 and 80%, with a yield of approximately 9 mg of protein measured using the Bradford assay (Bradford, 1976). YtxM appeared to be a monomer based on its elution volume on the Superdex-200 gel-filtration column.

A preliminary crystallization screen was carried out using the wild-type protein dialysed against 10 m*M* Tris–HCl pH 8.0 (at a concentration of ∼10 mg ml^{-1}) and the PEG/Ion Screen (Hampton Research) *via* the hanging-drop vapour-diffusion method (290 K). The crystallization conditions were subsequently optimized using the selenomethionine-incorporated protein dialysed against 0.1 *M* potassium formate, 20 m*M* Tris–HCl pH 8.0 and crystals grew optimally in 0.2 *M* potassium formate at pH 7.3 using a PEG 3350 range between 15 and 20%(*w*/*v*). These crystals had maximal dimensions of 0.2 × 0.2 × 0.05 mm and a plate-like morphology. For data collection, a single selenomethionine-incorporated YtxM crystal was stabilized in Paratone-N (Hampton Research) prior to flash-freezing at 100 K. Subsequently, a multiple-wavelength anomalous diffraction (MAD) experiment was carried out on a single selenomethionine-incorporated crystal to 2 Å resolution using an ADSC Quantum 4 CCD detector on ID14.4 at the ESRF, Grenoble, France (Fig. 2). Three wavelengths were chosen near the selenium absorption edge based on a fluorescence absorption spectrum obtained from the frozen crystal in order to maximize the f'' component (λ_1, peak), to minimize the f' component (λ_2, inflection) and to maximize $\Delta f'$ (λ_3, remote). The data for each wavelength were processed individually and scaled in such a way as to preserve the anomalous signal using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997). Details of the data-collection statistics are presented in Table 1.

Preliminary analysis of the X-ray diffraction data using the autoindexing routine in *DENZO* indicated that the crystal belonged to the orthorhombic system, point group 222, with unit-cell parameters a = 44.3, b = 90.9, c = 136.1 Å, $\alpha = \beta = \gamma = 90^\circ$ and a corresponding unit-cell volume of 5.48×10^5 Å^3. Consideration of the values of V_M suggest that the asymmetric unit contains two subunits, with a V_M value of 2.2 Å^3 Da^{-1} (Matthews, 1977). A self-rotation function showed no significant peaks. All axial reflections are present along h and absent when $l = 2n + 1$; however, no axial reflections were measured along k. Thus, one or possibly two axes are twofold screw axes, thereby identifying the space group as either $P222_1$ or $P2_12_12$ with the appropriate cyclic permutation of the axes. A full structural determination is currently under way.

Table 1
X-ray data-collection statistics for the selenomethionine-incorporated YtxM crystal collected on station ID 14.4 at the ESRF in Grenoble, France (20–2 Å resolution).

Values in parentheses refer to the highest resolution shell (2.05–2.0 Å).

Data set	Peak (λ_1)	Inflection (λ_2)	Remote (λ_3)
Wavelength (Å)	0.979451	0.979743	0.939281
Unique reflections	34397 (2153)	34145 (2131)	34125 (2119)
Multiplicity	5.4 (5.3)	5.2 (5.1)	5.4 (5.4)
Completeness (%)	91.0 (87.2)	90.0 (86.1)	90.3 (86.1)
$I/\sigma(I)$† (%)	85.6 (63.8)	85.6 (64.1)	83.8 (61.6)
R_{merge}‡	0.078 (0.258)	0.065 (0.280)	0.074 (0.330)

† The percentage of reflections with $I/\sigma(I)$ greater than three. ‡ $R_{merge} = \sum |I - \langle I \rangle| / \sum I$, where I is the integrated intensity of a given reflection.

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