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Crystallization and preliminary crystallographic analysis of the phosphotriesterase-like lactonase from *Geobacillus kaustophilus*

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GK1506 from the thermophilic bacterium *Geobacillus kaustophilus* is a member of the phosphotriesterase-like lactonases, which can catalyze the hydrolysis of a broad range of compounds with different chemical properties. It is of particular interest because of its high thermostability and its dual activity towards organophosphate compounds and some lactones. These properties make GK1506 an attractive target for future enzyme engineering and use in practical applications. In order to resolve the crystal structure of GK1506 and to gain a better understanding of its biological function, recombinant GK1506 was expressed, purified and crystallized using 0.1 *M* HEPES pH 7.6, 12%(*w*/*v*) PEG 8000, 8%(*v*/*v*) ethylene glycol at 291 K. A 2.6 Å resolution native data set was collected from a single flash-cooled crystal (100 K) using 10%(*v*/*v*) glycerol as a cryoprotectant. These crystals belonged to space group $P2_1$, with unit-cell parameters $a = 51.444$, $b = 80.453$, $c = 92.615$ Å, $\beta = 99.29^\circ$. Two molecules were assumed to be present per asymmetric unit, which gives a Matthews coefficient of 2.7 Å^3 Da^{-1}.

1. Introduction

Organophosphates (OPs) are well known potent toxic compounds that irreversibly inhibit acetylcholinesterase, a key enzyme of the central nervous system. Their toxic properties have been exploited for the development of chemical warfare agents and agricultural insecticides (Raushel, 2002). Phosphotriesterases (PTEs) that are capable of degrading these OPs are therefore attractive as potential antidotes for both organophosphate-based pesticides and nerve agents (LeJeune *et al.*, 1998). The phosphotriesterase from *Pseudomonas diminuta* is the most widely studied and characterized OP-degrading enzyme; it catalyzes the hydrolysis of the OP pesticides ethyl and methyl paraoxon (Aubert *et al.*, 2004). However, because OP compounds are synthetic, clues to the natural function of PTEs have been sparse until recently, when they were demonstrated to have promiscuous lactone-degrading activity (Afriat *et al.*, 2006). Following this discovery, other homologous enzymes such as those from *Deinococcus radiodurans* (*Dr*-OPH) and *Sulfolobus solfataricus* (SsoPox) have been isolated and shown to efficiently catalyze the hydrolysis of lactone compounds with promiscuous OP-degrading activity (Xiang *et al.*, 2009; Elias *et al.*, 2008). This activity places them into a newly emerging group of enzymes termed phosphotriesterase-like lactonases (PLLs). It has been hypothesized that phosphotriesterase enzymes evolved from lactonases. However, to date there have been no studies directed at establishing the phenotypes of OP-degrading enzyme knockout strains that would directly support the observed lactonase activity indeed being the natural function of the enzyme. One of the benefits of having enzymes that possess this dual substrate specificity is the potential for the development of practical applications that involve both remediation of OP-based toxic compounds and inhibition of quorum sensing, a process that relies on lactone signalling molecules. The ideal enzymes for use in these practical applications would possess high levels of solubility and thermostability (Vieille & Zeikus, 2001).

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Recently, we have identified and characterized an enzyme from *Geobacillus kaustophilus* (GK1506) that is homologous to members of the PTE family and have shown that it possesses a level of thermostability that greatly surpasses those of most thermophilic or mesophilic PTEs (unpublished work). For instance, the half-life ($t_{1/2}$) of GK1506 is about 7 h at 353 K, whereas *Dr*-OPH from *D. radiodurans* only remained active for 3 h at 333 K. Also, we have characterized its catalytic promiscuity toward OP compounds and some lactones in order to determine whether it contains the same dual activity as known PLL enzymes (unpublished work). In order to understand the structural basis of the thermostability of GK1506 and to improve its activity guided by the crystal structure, we report the crystallization and preliminary crystallographic studies of GK1506.

2. Cloning, expression and purification

The DNA fragment for the GK1506 gene was amplified by PCR from the genomic DNA of the thermophilic bacterium *G. kaustophilus* HTA426 using *Ex Taq* DNA polymerase (Takara). The primers 5′-GCGC**GGATCC**ATGGCGGAGATGGTAGAAACGGTAT-3′ and 5′-GATC**AAGCTT**GTCAAGCCGAGAACAGCGCCGCCGGAT-3′ were used. The sequences recognized by the upstream *Bam*HI restriction enzyme and downstream *Hin*dIII restriction enzyme are shown in bold. The PCR conditions were 368 K for 5 min followed by 30 cycles of 368 K for 1 min, 329 K for 1 min and 345 K for 2 min and final extension at 345 K for 10 min. The PCR product was digested with *Bam*HI and *Hin*dIII, purified using an AxyPrep PCR Clean-up Kit (Axygen, USA) and ligated into similarly digested expression vector pET28a. The ligation product was transformed into *Escherichia coli* XL1-blue electrocompetent cells and plated onto an LB agar plate (containing 50 µg ml^{-1} kanamycin) overnight. The correct sequence was confirmed by DNA sequencing. The recombinant plasmid pET28a6His-GK1506 encodes an additional 34-amino-acid N-terminal sequence containing a six-histidine tag.

The recombinant plasmid was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL electrocompetent cells. Cells were grown at 310 K in LB medium supplemented with 50 µg ml^{-1} kanamycin and 1.0 m*M* $CoCl_2$ until the OD_{600} reached 0.6–0.8; protein expression was induced by the addition of 1.0 m*M* IPTG and the culture was grown for 12 h at 299 K. Cells were harvested by centrifugation and resuspended in 50 m*M* Tris–HCl pH 8.0 containing 0.2 m*M* Co^{2+} and 5 m*M* imidazole. After ultrasonic cell disintegration, the cell suspension was centrifuged at 8000 rev min^{-1} for 10 min. The cytosolic fraction was heated to 333 K for 30 min and centrifuged in order to remove heat-induced aggregated proteins. The supernatant was loaded onto a nickel-chelating chromatography column and eluted with a gradient from 20 to 100 m*M* imidazole in 20 m*M* Tris–HCl pH 7.9, 0.5 *M* NaCl. Resource Q anion-exchange chromatography (GE Healthcare, USA) was subsequently applied using a 0–1 *M* gradient of NaCl in 20 m*M* Tris–HCl pH 8.0 buffer. The target protein was finally eluted with approximately 0.2 *M* NaCl. The purity of GK1506 was estimated to be greater than 99% by SDS–PAGE analysis.

3. Crystallization

The purified GK1506 protein was concentrated to 5, 10 and 20 mg ml^{-1} in 10 m*M* Tris–HCl pH 8.0, 10 m*M* NaCl. Crystallization was performed by the hanging-drop vapour-diffusion method at 291 K in 16-well plates. Each drop consisted of 1 µl protein solution and 1 µl reservoir solution, with 200 µl reservoir solution in the well. Screening was carried out with Hampton Research Crystal Screen kits and positive hits were then optimized. Initial crystals were obtained from condition No. 37 of Crystal Screen [0.1 *M* HEPES pH 7.5, 10%(*w*/*v*) PEG 8000, 8%(*v*/*v*) glycol]. A large number of very small crystals grew in two weeks from the pellucid solution and showed poor X-ray diffraction quality. Further crystallization optimization was performed by carefully adjusting the concentrations of PEG 8000 (6–13%) and glycol (6–12%) and the buffer pH value (6–9) together with the protein concentration (5–25 mg ml^{-1}). Fortunately, single crystals were obtained from an optimized reservoir solution [0.1 *M* HEPES pH 7.6, 12%(*w*/*v*) PEG 8000, 8%(*v*/*v*) glycol] within a month with dimensions of 0.3 × 0.1 × 0.05 mm (Fig. 1) and diffracted to 2.6 Å resolution on the home Rigaku MicroMax-007 X-ray source (Fig. 2).

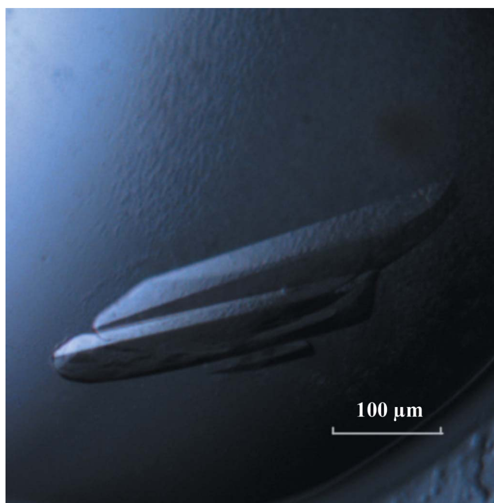

Figure 1
Typical crystals of GK1506.

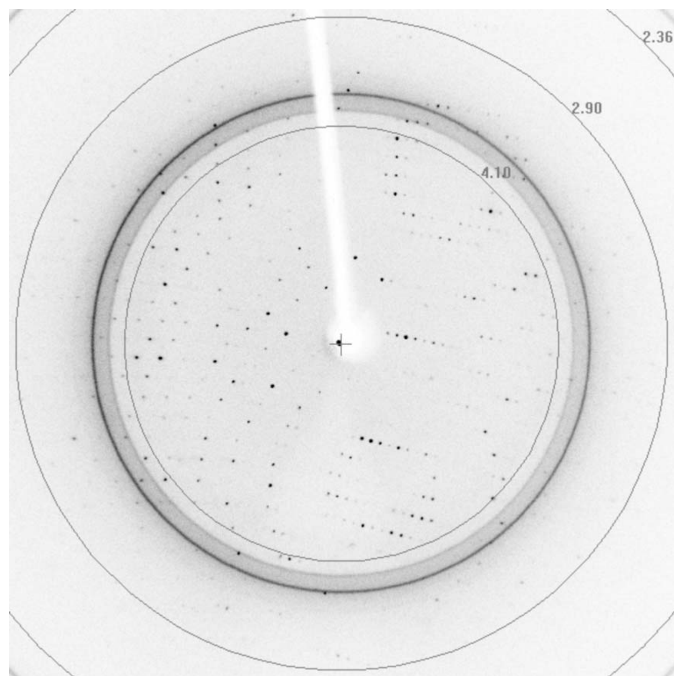

Figure 2
X-ray diffraction image of GK1506.

Table 1
Data-collection and processing statistics for GK1506.

Values in parentheses are for the highest resolution shell.

Unit-cell parameters (Å, °)	a = 51.4, b = 80.5, c = 92.6, β = 99.3
Space group	$P2_1$
Wavelength (Å)	1.5418
Resolution (Å)	50.0–2.6 (2.70–2.60)
Total No. of reflections	253674 (24512)
No. of unique reflections	23055 (2201)
Completeness (%)	100.0 (95.2)
Average $I/\sigma(I)$	7.6 (3.2)
$R_{\rm merge}$† (%)	9.5 (43.6)
Molecules per asymmetric unit	2
$V_{\rm M}$ (Å^3 Da^{-1})	2.7
Solvent content (%)	32

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the observations $I_i(hkl)$ of reflection hkl.

4. Data collection and processing

Native diffraction data were collected on a MAR345dtb (MAR Research, Hamburg) image-plate detector at 100 K using a Rigaku MicroMax-007 rotating-anode home X-ray generator operating at 40 kV and 20 mA (λ = 1.5418 Å). The crystal was mounted on a nylon loop and flash-cooled in a nitrogen-gas cryostream at 100 K using an Oxford Cryosystem. Crystals were cryoprotected by the addition of 10%(v/v) glycerol to the crystallization conditions. A total of 180 frames of data were collected with a 1° oscillation range. All intensity data were indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). The crystal belonged to space group $P2_1$, with unit-cell parameters a = 51.4, b = 80.5, c = 92.6 Å, β = 99.29°. We assumed the presence of two molecules per asymmetric unit, which gives a Matthews coefficient of 2.7 Å^3 Da^{-1} and 32% solvent content (Matthews, 1968). Complete data-collection statistics are given in Table 1. Molecular replacement was performed using the solution structure of organophosphate hydrolase from *D. radiodurans* (*Dr*-OPH; PDB entry 3htw; Hawwa *et al.*, 2009) as the initial search model, which showed 60% sequence similarity to GK1506. This procedure was performed using *CNS* v.1.2 (Brünger *et al.*, 1998) and *Phaser* (McCoy *et al.*, 2007). Structure determination and refinement is ongoing.

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