Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Purification, crystallization and preliminary X-ray analysis of the peptidoglycan *N*-acetylglucosamine deacetylase BC1960 from *Bacillus cereus* in the presence of its substrate $(GlcNAc)_6$

Aleka Tsalafouta,[a] Emmanuel Psylinakis,[b] Evangelia G. Kapetaniou,[b] Dina Kotsifaki,[b] Alexandra Deli,[a] Alexandros Roidis,[b] Vasilis Bouriotis[a,b] and Michael Kokkinidis[a,b]*

[a]Department of Biology, University of Crete, PO Box 2208, GR-71110, Heraklion, Crete, Greece, and [b]Institute of Molecular Biology and Biotechnology (IMBB), PO Box 1527, GR-71110, Heraklion, Crete, Greece

Correspondence e-mail: kokkinid@imbb.forth.gr

Received 2 November 2007
Accepted 23 January 2008

The peptidoglycan *N*-acetylglucosamine (GlcNAc) deacetylase BC1960 from *Bacillus cereus* (EC 3.5.1.33), an enzyme consisting of 275 amino acids, was crystallized in the presence of its substrate $(GlcNAc)_6$. The crystals belonged to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 92.7$, $c = 242.9$ Å and four molecules in the asymmetric unit. A complete data set was collected at 100 K to a resolution of 2.38 Å using synchrotron radiation.

1. Introduction

Polysaccharide deacetylases belong to carbohydrate esterase family 4 (CE4), which includes chitin deacetylases, acetylxylan esterases, xylanases, rhizobial NodB chitooligosaccharide deacetylases and peptidoglycan deacetylases (Coutinho & Henrissat, 1999). Sequence alignments reveal that all CE4-family members contain a highly conserved catalytic core, termed the NodB-homology domain (Blair & van Aalten, 2004). All members of this family catalyze the hydrolysis of either N-linked acetyl groups from *N*-acetylglucosamine residues (chitin deacetylases, NodB chitooligosaccharide deacetylases and peptidoglycan *N*-acetylglucosamine deacetylases) or O-linked acetyl groups from *O*-acetylxylose residues (acetylxylan esterases, xylanases) (Caufrier *et al.*, 2003). It has been suggested that the members of the CE4 family are metal-dependent enzymes, since the addition of divalent cations results in increased enzymatic activity (Psylinakis *et al.*, 2005; Blair & van Aalten, 2004).

The bacterial cell wall, which consists mainly of peptidoglycan, plays a vital role in maintenance of the stability and viability of bacterial cells. Different kinds of modification of the peptidoglycan, such as *N*-acetylglucosamine (GlcNAc) deacetylation, play a crucial role in a number of bacterial biological functions such as bacterial growth, division and autolysis (Boneca *et al.*, 2005). The sequencing of the *Bacillus cereus* genome revealed ten open reading frames for putative polysaccharide deacetylases, six of which have been proposed to encode putative peptidoglycan deacetylases (Fukushima *et al.*, 2002). In order to gain insight into the structural basis of peptidoglycan *N*-acetylglucosamine deacetylation, we have initiated structural studies of the BC1960 peptidoglycan GlcNAc deacetylase from *B. cereus*. The enzyme (MW 30 570 Da) exhibits very high homology (94% identity) to a putative polysaccharide deacetylase from *B. anthracis*. BC1960 is a thermally stable enzyme with an optimum temperature for activity at 323 K, an optimum pH of 6.0 and is dependent on Co^{2+} ions for enzymatic activity. It deacetylates *N*-acetylchitooligosaccharides $(GlcNAc)_{2-6}$ of the cell-wall peptidoglycan, with $(GlcNAc)_4$ being the favoured substrate (Psylinakis *et al.*, 2005).

Some microorganisms (*e.g.* *Staphylococcus aureus*, *Neisseria meningitidis* and *Mycobacterium tuberculosis*) use peptidoglycan modifications as a means to counteract the activity of the host lysozyme, which suggests a probable biological role of peptidoglycan deacetylases in the protection of bacteria from the defence mechanisms of the hosts (Boneca *et al.*, 2007). Structural studies of

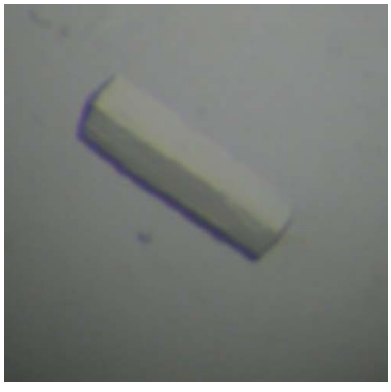

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	$a = b = 92.7$, $c = 242.9$
Matthews coefficient (Å^3 Da^{-1})	2.14
Resolution (Å)	30–2.38 (2.47–2.38)
Total observations	9256092
Unique reflections	43221 (4197)
Data completeness (%)	99.6 (99.5)
$R_{\rm merge}$† (%)	0.098 (0.5)
Average $I/\sigma(I)$	21.3 (5.2)
Mosaicity (°)	1.3

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

the peptidoglycan deacetylase BC1960 from *B. cereus* may lead to drug design that will probably also be relevant to the homologous enzyme from *B. anthracis*, a pathogen which is considered to be a potential bioweapon.

2. Materials and methods

2.1. Expression and purification

The *bc1960* gene encoding BC1960 (accession code Q81EK9) was cloned, inserted into pET-26b vector (Novagen) containing a C-terminal 6×His tag and transformed into *Escherichia coli* strain BL21 (DE3) (Psylinakis *et al.*, 2005). A sufficient amount of soluble protein (without the N-terminal signal sequence of eight amino acids) was obtained after expression using the following conditions. Cells were grown in 2 l LB medium containing 34 µg ml^{-1} chloramphenicol and 30 µg ml^{-1} kanamycin at 298 K until OD_{600} reached 0.7. The culture was induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 298 K and harvested by centrifugation at 6370*g* for 30 min at 277 K. The precipitated cells were resuspended in 50 ml lysis buffer containing 25 m*M* Tris pH 8, 300 m*M* NaCl, 5 m*M* imidazole, 15 m*M* β-mercaptoethanol, 1 m*M* PMSF, 20 µg ml^{-1} leupeptin and 150 µg ml^{-1} benzamidine and homogenized by sonication. The precipitate was removed by centrifugation at 18 500*g* for 1 h at 279 K. Purification was effected *via* the His tag by affinity chromatography at 277 K on a 10 ml Ni–NTA chelating column (Qiagen) pre-equilibrated in lysis buffer. The column was washed with ten column volumes of 5 m*M* imidazole and five column volumes of 10 m*M* imidazole, followed by a gradient from 20 to 250 m*M* imidazole. BC1960 started to elute at 200 m*M* imidazole. Fractions containing more than 80% homogeneous BC1960, as shown by SDS–PAGE gels (MW 30 570 Da), were pooled and dialyzed extensively against 25 m*M* Tris–HCl pH 7.5 containing 200 m*M* NaCl and 15 m*M* β-mercaptoethanol. The protein solution was concentrated using Amicon Ultra-15 filters. Sephacryl S-200 size-exclusion chromatography was performed with 25 m*M* Tris–HCl pH 7.5 containing 200 m*M* NaCl and 15 m*M* β-mercaptoethanol (XK26/60 column, Amersham Pharmacia Biotech). BC1960 elutes as a single peak after 160–190 ml of this buffer. Typical yields are 30 mg homogeneous BC1960 from approximately 9.5 g cell paste. Prior to crystallization, BC1960 was concentrated to 10 mg ml^{-1} in 25 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl and 15 m*M* β-mercaptoethanol using Amicon Ultra-15 filters and incubated with 3.75 m*M* $(GlcNAc)_6$.

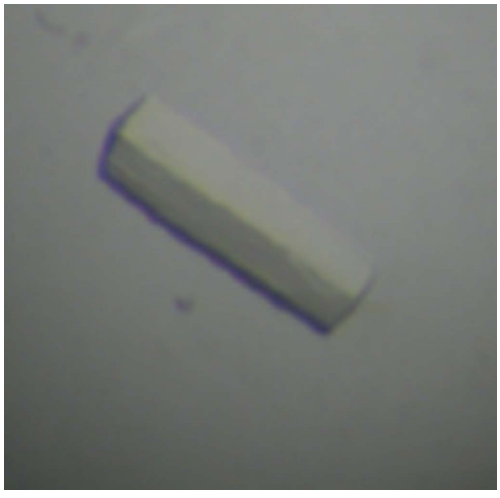

Figure 1
Crystal of the BC1960–$(GlcNAc)_6$ complex. The approximate length of the crystal is 0.9 mm.

2.2. Crystallization

Cocrystallization experiments of BC1960 with *N*-acetychitooligosaccharides $(GlcNAc)_{2–6}$ produced data-quality crystals using $(GlcNAc)_6$. Initial crystallization conditions for BC1960 in the presence of $(GlcNAc)_6$ were determined by the hanging-drop vapour-diffusion method using Crystal Screen (Hampton Research) and 24-well Linbro cell-culture plates. The hanging drops were made up of a 3 µl protein–substrate mixture to which 3 µl reservoir solution was added. The drops were equilibrated against 1000 µl reservoir solution at room temperature. After refinement of the initial conditions, crystals of reasonable size for crystallographic studies were obtained with 12–18%(*w*/*v*) PEG 4000, 0.1 *M* Tris acetate pH 4.6, 0.2 *M* ammonium sulfate, 3.75 m*M* $(GlcNAc)_6$ and 1 m*M* EDTA (Fig. 1). EDTA was used to prevent processing of the substrate by the metal-dependent enzyme.

2.3. Data collection and processing

X-ray diffraction data were collected using synchrotron radiation. A native data set was collected from a single crystal on the EMBL BW7A beamline at the DORIS storage ring, DESY, Hamburg. Crystals were flash-cooled to 100 K in a nitrogen-gas cold stream using an Oxford Cryosystems device. 999 images with 0.2° rotation each were collected to a resolution of 2.38 Å using a wavelength of 0.817 Å. Diffraction data were recorded on a MAR CCD detector with a diameter of 225 mm. Data were processed and scaled with *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997). The Matthews coefficient (Matthews, 1968) was calculated with the program *MATTHEWS_COEF* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The diffraction data from the BC1960 crystal are consistent with the tetragonal space groups $P4_12_12$ or $P4_32_12$. Data-collection and processing statistics are given in Table 1. The overall redundancy of the data is 13. Assuming the presence of four molecules in the asymmetric unit, the Matthews coefficient is 2.14 Å^3 Da^{-1} and the solvent content is 42.5%.

We thank the European Molecular Biology Laboratory, Hamburg Outstation and the European Union for support through the EU-I3

access grant from the EU Research Infrastructure Action under the FP6 'Structuring the European Research Area Programme', contract No. RII3/CT/2004/5060008. Expert assistance by Dr Manfred Weiss is gratefully acknowledged. Partial support was provided by the PEP and Pythagoras programmes of the GSRT.

References

Blair, D. E. & van Aalten, D. M. F. (2004). *FEBS Lett.* **570**, 13–19.
Boneca, I. G. (2005). *Curr. Opin. Microbiol.* **8**, 46–53.
Boneca, I. G. *et al.* (2007). *Proc. Natl Acad. Sci. USA*, **104**, 997–1002
Caufrier, F., Martinou, A., Dupont, C. & Bouriotis, V. (2003). *Carbohydr. Res.* **338**, 687–693.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Coutinho, P. M. & Henrissat, B. (1999). *Carbohydrate Active Enzymes: An Integrated Database Approach*, edited by H. J. Gilbert, G. Davies, B. Henrissat & B. Svensson, pp. 3–12. Cambridge: The Royal Society of Chemistry.
Fukushima, T., Yamamoto, H., Atrih, A., Foster, S. J. & Sekiguchi, J. (2002). *J. Bacteriol.* **184**, 6007–6015.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Psylinakis, E., Boneca, I. G., Mavromatis, K., Deli, A., Hayhurst, E., Foster, S., Varum, K. & Bouriotis, V. (2005). *J. Biol. Chem.* **280**, 30856–30863.