Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of cellobiose phosphorylase from *Cellvibrio gilvus*

Masafumi Hidaka,[a] Motomitsu Kitaoka,[b] Kiyoshi Hayashi,[b] Takayoshi Wakagi,[a] Hirofumi Shoun[a] and Shinya Fushinobu[a]*

[a]Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunko-ku, Tokyo 113-8657, Japan, and [b]National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

Correspondence e-mail: asfushi@mail.ecc.u-tokyo.ac.jp

Received 4 June 2004
Accepted 20 July 2004

A recombinant cellobiose phosphorylase from *Cellvibrio gilvus* has been prepared and crystallized by the sitting-drop vapour-diffusion method using 10 mg ml^{-1} purified enzyme, 1.5 *M* ammonium sulfate, 0.1 *M* MES buffer pH 7.0 and 5 m*M* glucose. A suitable crystal was obtained after 10 d incubation at 298 K. The crystal belongs to space group $P2_1$, with unit-cell parameters $a = 84.77$, $b = 98.31$, $c = 104.04$ Å, $\beta = 102.73°$. X-ray diffraction data to 2.1 Å resolution have been collected at KEK-PF BL-5A.

1. Introduction

Sugar phosphorylases catalyze the phosphorolysis of glycosidic bonds to generate glycosyl phosphates. Since the energy of a glycosyl phosphate bond is not as high as that of a glycosyl nucleotide bond (the substrate for glycosyl transferases), the reaction is reversible. Therefore, sugar phosphorylases can be employed for both the synthesis and degradation of sugar chains, exploiting the reversible reaction.

Cellobiose phosphorylase (CBP; EC 2.4.1.20) catalyzes the reversible phosphorolysis of cellobiose to α-glucose-1-phosphate and glucose (Sih & McBee, 1955). This enzyme is present as an intracellular enzyme in bacterial cells that utilize cellulose (Ayers, 1958; Hulcher & King, 1958; Sato & Takahashi, 1967; Yernool *et al.*, 2000). CBP is thought to be involved in the utilization of extracellular cellobiose formed through the action of cellulase. CBP has been classified into glycosyl transferase (GT) family GT-36 based on amino-acid sequence similarity and on the finding that the enzyme shows no hydrolytic activity (Coutinho *et al.*, 2003). Some sugar phosphorylases [*e.g.* cellodextrin phosphorylase (CDP) and chitobiose phosphorylase (ChBP)] have also been classified into this family. In spite of the 40% amino-acid sequence identity between CBP from *Cellvibrio gilvus* (CgCBP) and CDP from *Clostridium stercorarium*, their substrate specificities with regard to the degree of polymerization are quite different: CBP shows activity only toward disaccharides, whereas CDP phosphorolyses cellotriose and higher cellooligosaccharides (Reichenbecher *et al.*, 1997). The three-dimensional structure of ChBP from *Vibrio proteolyticus* has been determined to be an $(\alpha/\alpha)_6$-barrel fold by X-ray crystallographic analysis (Hidaka *et al.*, 2004). While the structure of ChBP from *V. proteolyticus* showed no structural similarity to those of any other glycosyl transferases, notable structural similarities were found with members of glycoside hydrolase families GH-15 and GH-65. In addition, significant similarities in their reaction mechanisms were indicated. These findings led to the reclassification of CBP into a novel GH family, GH-94.

CgCBP is one of the most studied GH-94 enzymes (Kitaoka *et al.*, 1992*a*,*b*, 1993; Kitaoka & Hayashi, 2001; Liu *et al.*, 1998; Percy, Ono & Hayashi, 1998; Percy, Ono, Watt *et al.*, 1998). The phosphorolytic reaction was found to proceed *via* an ordered Bi–Bi mechanism (Kitaoka *et al.*, 1992*a*,*b*). The order of substrate binding and release of products was determined to be as follows: cellobiose initially bound to the enzyme, followed by phosphate; glucose was then released and finally glucose-1-phosphate was released to complete the reaction. Interestingly, the substrate-binding order was different from that in the case of CBP from *Clostridium thermocellum* YM4, although the enzymes showed a high level of amino-acid sequence identity (63%; Kim *et al.*, 2002). CgCBP showed a low level of identity with chitobiose phosphorylase (33%; Honda *et al.*, 2004). The three-dimensional structure of CgCBP will be helpful for clarifying its reaction mechanism and will be useful for further protein engineering and rational design for industrial use. In this report, we describe the crystallization of CgCBP and preliminary X-ray diffraction data collection.

2. Experimental procedures and results

2.1. Preparation of the expression plasmid and the recombinant enzyme

The CgCBP gene was amplified by the polymerase chain reaction method using KOD-Plus polymerase (Toyobo, Osaka, Japan) and the two oligonucleotide primers GGC ACC

© 2004 International Union of Crystallography
Printed in Denmark – all rights reserved

 DOI: 10.1107/S0907444904017767

ACA TAT GCG GTA CGG CCA TTT CGA C and GGA CC**A AGC TT**T CAG ACG GTC ACC TCG ACG C, where the sequences in bold represent the restriction-endonuclease sites. The amplified fragment was inserted between the *Nde*I and *Hin*dIII sites of expression vector pET28a (Novagen, Darmstadt, Germany) to construct an expression vector with a 6×His sequence added to its N-terminus. The vector was transformed into *Escherichia coli* strain BL21(DE3). The resultant transformants were incubated in Luria–Bertani (LB) medium containing 50 mg l^{-1} kanamycin at 310 K until the optical density at 660 nm reached 0.6. Isopropyl 1-thio-β-D-galactoside was then added to a final concentration of 0.1 m*M* and the cultures were incubated further for 24 h at 310 K. The cells were harvested by centrifugation at 10 000*g* for 10 min and then resuspended in 20 m*M* sodium phosphate buffer pH 7.5 containing 0.5 *M* NaCl (buffer *A*). The resuspended cells were then sonicated and the cell debris was removed by centrifugation at 17 000*g* for 60 min. The cell-free extract was loaded onto an Ni–NTA agarose (Qiagen, Hilden, Germany) column and the enzyme was eluted with a stepwise gradient of imidazole (10, 20 and 500 m*M*) in buffer *A*. Appropriate fractions were dialyzed against 5 m*M* Tris–HCl pH 7.5 and then concentrated (10 mg ml^{-1}). The homogeneity of the purified enzyme was established by SDS–PAGE analysis, while the protein concentration was determined from the absorbance at 280 nm based on the theoretical molar coefficient (161 321 M^{-1} cm^{-1}) determined from the amino-acid composition of CgCBP.

2.2. Crystallization

The initial crystallization conditions were screened by the sitting-drop vapour-diffusion method with Crystal Screen 1 and 2 crystallization kits (Hampton Research, Laguna Niguel, CA, USA) at 278 and 298 K. Each drop was prepared by mixing 2.0 µl purified enzyme solution with the same volume of reservoir solution and was equilibrated against 100 µl reservoir solution. Crystals of CgCBP were obtained in drops of solution No. 23 of Crystal Screen 2 [1.6 *M* ammonium sulfate, 0.1 *M* MES pH 6.5 and 10%(*v*/*v*) dioxane] in six months. To increase the crystallization rate, the condition was improved by the addition of 5 m*M* glucose and the omission of dioxane. Clusters of thin needle-shaped crystals grew in less than 10 d, but were unsuitable for X-ray analysis. To obtain suitable crystals for data collection, the pH was optimized by changing the buffer. The best crystallization conditions were obtained with a reservoir solution comprising 1.5 *M* ammonium sulfate, 0.1 *M* MES pH 7.0 and 5 m*M* glucose at 298 K. The optimum conditions resulted in the formation of rod-like crystals (0.1 × 0.1 × 0.3 mm) in less than 10 d (Fig. 1).

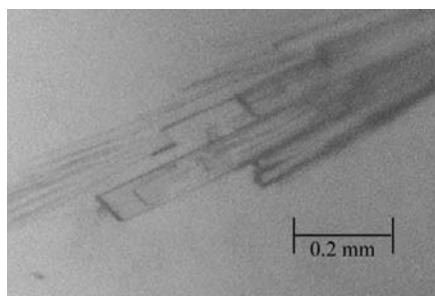

Figure 1
Crystals of CgCBP.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Wavelength (Å)	1.0000
Beamline	PF BL-5A
Space group	$P2_1$
Unit-cell parameters	
a (Å)	84.77
b (Å)	98.31
c (Å)	104.04
β (°)	102.73
Resolution (Å)	72.55–2.10 (2.21–2.10)
Measured reflections ($I > 3\sigma$)	348 528
Unique reflections	94 231
Completeness (%)	97.2 (95.5)
Redundancy	3.7 (3.5)
Mean $I/\sigma(I)$	8.1 (2.5)
$R_{\rm merge}$† (%)	7.4 (29.2)

† $R_{\rm merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the *i*th measurement of reflection *h* and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all *i* measurements.

2.3. Data collection

Diffraction data for CgCBP were collected at beamline BL-5A, a new multi-pole wiggler beamline at the Photon Factory, High Energy Accelerator Research Organization (KEK), Tsukuba, Japan (λ = 1.0000 Å). CgCBP crystals were transferred to a reservoir solution containing 20%(*w*/*v*) glycerol and then flash-cooled in a stream of liquid nitrogen at 100 *K*. The X-ray diffraction data were collected using an ADSC Quantum 315 CCD X-ray detector. A total of 180 frames were collected with 1.0° oscillations and 10 s exposures. The data were processed and scaled using *MOSFLM* (Leslie, 1992).

The crystal of CgCBP belongs to the monoclinic space group $P2_1$ and diffracted to 2.1 Å resolution. The data statistics are presented in Table 1. Assuming two molecules of CgCBP per asymmetric unit, the V_M value (Matthews, 1968) and solvent content were determined to be 2.4 Å^3 Da^{-1} and 48.0%, respectively. Initial phases were successfully obtained by the molecular-replacement method, using the structure of chitobiose phosphorylase from *V. proteolyticus* (PDB code 1v7w) as a search model. Crystallographic refinement is currently in progress.

We wish to thank Drs M. Suzuki, N. Igarashi, N. Matsugaki and S. Wakatsuki for data collection at the Photon Factory, KEK, Japan. This work was supported by the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid for Scientific Research (15780067) to SF and Research Fellowships from the JSPS for Young Scientists (15-11327) to MH. This work was also supported in part by the National Project on Protein Structural and Functional Analysis and by the SBSP (Structural Biology Sakabe Project), FAIS (Foundation for Advancement of International Science).

References

Ayers, W. A. (1958). *J. Bacteriol.* **76**, 515–517.
Coutinho, P. M., Deleury, E., Davies, G. J. & Henrissat, B. (2003). *J. Mol. Biol.* **328**, 307–317.
Hidaka, M., Honda, Y., Kitaoka, M., Nirasawa, S., Hayashi, K., Wakagi, T., Shoun, H. & Fushinobu, S. (2004). *Structure*, **12**, 937–947.
Honda, Y., Kitaoka, M. & Hayashi, K. (2004) *Biochem. J.* **377**, 225–232.
Hulcher, F. H. & King, K. W. (1958). *J. Bacteriol.* **76**, 571–577.
Kim, Y. K., Kitaoka, M., Krishnareddy, M., Mori, Y. & Hayashi, K. (2002). *J. Biochem.* **132**, 197–203.
Kitaoka, M. & Hayashi, K. (2001). *Trends Glycosci. Glycotechnol.* **14**, 35–50.
Kitaoka, M., Ogawa, S. & Taniguchi, H. (1993). *Carbohydr. Res.* **247**, 355–359.
Kitaoka, M., Sasaki, T. & Taniguchi, H. (1992*a*). *Biosci. Biotechnol. Biochem.* **56**, 652–655.
Kitaoka, M., Sasaki, T. & Taniguchi, H. (1992*b*). *J. Biochem.* **112**, 40–44.
Leslie, A. G.. W. (1992). *Jnt CCP4–ESF/EACBM Newsl. Protein Crystallogr.* **26**.
Liu, A., Tomita, H., Li, H., Miyaki, H., Aoyagi, C., Kaneko, S. & Hayashi, K. (1998). *J. Ferment. Bioeng.* **85**, 511–513.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Percy, A., Ono, H. & Hayashi, K. (1998). *Carbohydr. Res.* **308**, 423–429.
Percy, A., Ono, H., Watt, D. & Hayashi, K. (1998). *Carbohydr. Res.* **305**, 543–548.
Reichenbecher, M., Lottspeich, F. & Bronnenmeier, K. (1997). *Eur. J. Biochem.* **247**, 262–267.
Sato, M. & Takahashi, H. (1967). *Agric. Biol. Chem.* **31**, 470–474.
Sih, C. J. & McBee, R. H. (1955). *Proc. Montana Acad. Sci.* **15**, 21–22.
Yernool, D. A., McCarthy, J. K., Eveleigh, D. E. & Bok, J.-D. (2000). *J. Bacteriol.* **182**, 5172–5179.