Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of the 6-phospho-α-glucosidase from *Bacillus subtilis*

Annabelle Varrot,[a] Hiroki Yamamoto,[b] Junichi Sekiguchi,[b] John Thompson[c] and Gideon J. Davies[a]*

[a]Department of Chemistry, University of York, Heslington, York YO10 5DD, England, [b]Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda-shi, Nagano 386, Japan, and [c]Microbial Biochemistry and Genetics Unit, Oral Infection and Immunity Branch, NIDR, Bethesda, Maryland 20892, USA

Correspondence e-mail: davies@yorvic.york.ac.uk

Received 15 February 1999
Accepted 8 March 1999

6-Phospho-α-glucosidase (GlvA) is the protein involved in the dissimilation of α-glycosides accumulated *via* a phospho*enol*pyruvate-dependent maltose phosphotransferase system (PEP-PTS) in *Bacillus subtilis*. The purified enzyme has been crystallized in a form suitable for X-ray diffraction analysis. Thin rod-like crystals have been grown by the hanging-drop method in the presence of manganese and NAD. They diffract beyond 2.2 Å using synchrotron radiation and belong to the space group *I*222 (or its enantiomorph) with unit-cell dimensions a = 83.26, b = 102.56, c = 145.31 Å and contain a single molecule of GlvA in the asymmetric unit.

1. Introduction

The Gram-positive spore-forming organism *Bacillus subtilis* accumulates carbohydrates in its cytoplasm *via* a phospho*enol*pyruvate-dependent sugar phosphotransferase system (PEP-PTS). These group-transfer systems are found in various species of both Gram-positive (Hengstenberg *et al.*, 1989; Meadow *et al.*, 1990; Reizer *et al.*, 1988) and Gram-negative bacteria (Hengstenberg *et al.*, 1993; Postma *et al.*, 1993). The multimolecular PEP-PTS, first described by Roseman and colleagues, permits simultaneous transport and phosphorylation of sugar across the bacterial membrane (Kundig *et al.*, 1964; Roseman, 1989). The general cytoplasmic enzymes, named Enzyme I and HPr, together with the sugar-specific enzymes IIA and IIB(C) (nomenclature reviewed in Saier & Reizer, 1992) permit the transfer of the high-energy phosphoryl group from PEP to the incoming sugar. Enzyme IIB is localized in the membrane and it recognizes, binds and simultaneously phosphorylates specific sugars during transit across the cell membrane. The disaccharide–phosphates must then be cleaved to the appropriate free and phosphorylated sugar moieties in order to enter into the energy-generating pathways of the bacteria. This function is performed by specific phosphoglycoside hydrolases, including 6-phospho-β-glucosidase (Wilson & Fox, 1974) and 6-phospho-β-galactosidase (Hengstenberg *et al.*, 1993).

The 449 amino-acid 6-phospho-α-glucosidase GlvA participates in the catabolism of phosphorylated α-glucosides produced *via* a maltose PEP-PTS. In the *B. subtilis* genome, the *glvA* gene (formerly *glv1*) is encoded within the maltose PEP-PTS operon (Kunst *et al.*, 1997; Yamamoto *et al.*, 1996), as is often the case for phosphoglycoside hydrolases. GlvA shows 74 and 78% identity with the 6-phospho-α-glucosidase MalH from *Fusobacterium mortiferum* and GlvG from *Escherichia coli*, respectively (Bouma *et al.*, 1997; Reizer *et al.*, 1994). It has been assigned to family 4 of the glycoside hydrolases (Henrissat & Bairoch, 1993; Thompson *et al.*, 1998), although whether its mechanism is that of a true glycoside hydrolase is open to doubt (see below). The natural substrate of GlvA is maltose-6-phosphate, which is hydrolysed to glucose-6-phosphate and glucose. GlvA can also cleave trehalose-6-phosphate to yield the same reaction products. Enzyme activity requires both NAD(H) and a divalent metal, such as Mn^{2+}, as does activity for three other family 4 members, namely, MalH from *F. mortiferum*, CelF (6-phospho-β-glycosidase) from *E. coli* (Parker & Hall, 1990; Thompson *et al.*, 1995) and α-galactosidase from *E. coli* (Burstein & Kepes, 1971). Analytical centrifugation experiments show that the divalent metal has at least a structural role and that Mn^{2+} permits formation of the active tetramer of GlvA from its inactive dimeric state. The role of NAD(H) has not yet been clarified.

The three-dimensional fold and catalytic mechanism of this family, which is composed only of proteins of bacterial origin, is not known. Indeed, whether the members of this family are true glycoside hydrolases (reviewed in Henrissat & Davies, 1997) is a matter of some debate, since they display characteristics which are unique to this family. For example, both equatorial and axial substrates are cleaved and the enzymes require NAD(H) for catalysis. Two residues, Asp41 and Glu359, are invariant throughout family 4, whilst a glutamyl or aspartyl residue is always found at position 111, implicating these three acidic

residues in catalysis. Asp41 is found at the N-terminus region of the protein, in a G*x*GSx_{25}D motif which may represent the dinucleotide-binding region. The two glutamates Glu111 and Glu359 are potential catalytic residues in GlvA from *B. subtilis*. Indeed, their mutation leads to inactivation of the enzyme (Thompson *et al.*, 1998).

Classical glycoside hydrolases perform the cleavage of the glycosidic bond with general acid/base catalytic assistance, which may lead to either retention or inversion of the anomeric configuration (Koshland, 1953; Sinnott, 1990). Both retention and inversion mechanisms require two enzymatic carboxylates. The determination of the GlvA structure will first reveal if the catalysis is performed *via* general acid/base catalysis or whether the enzyme has evolved a novel mechanism for the cleavage of glycosidic bonds such as an NAD(H)-catalyzed lyase mechanism. Here, we report the preliminary crystallographic analysis of the 6-phospho-α-glucosidase GlvA from *B. subtilis* in an NAD/Mn^{2+}-dependent crystal form. Solution of the structure of GlvA should shed light on the unanswered mechanistic questions.

2. Experimental

2.1. Crystallization

The 6-phospho-α-glucosidase gene (*glvA*) from *B. subtilis* has been cloned from *B. subtilis* and overexpressed in *E. coli* as described previously (Thompson *et al.*, 1998). Pure protein, as judged by electrophoresis, was washed on Filtron Microsep 10K membranes with a buffer containing 10 m*M* HEPES pH 7.0, 2 m*M* $MnSO_4$ and 0.1 m*M* NAD and concentrated to 10 mg ml^{-1}. Crystallization was performed by vapour-phase diffusion using the hanging-drop technique in Falcon 3047 multiwell plates at 291 K. Drops were obtained by mixing 1 µl the protein solution with an equal volume of reservoir solution. Initial crystallization conditions were determined following a sparse-matrix screening approach (Jancarik & Kim, 1991). Optimized crystallization trials of *B. subtilis* GlvA produced long thin rods or occasionally plates in 3–5 d. The rods have typical dimensions of approximately 0.05 × 0.05 × 0.3 mm (Fig. 1) and the plates have approximate dimensions 0.3 × 0.15 × 0.02 mm. Crystals were obtained with a solution containing 100 m*M* HEPES pH 7.5 as buffer, 1.15 *M* trisodium citrate as precipitant and 20 m*M* $MnSO_4$. To facilitate cryocrystallographic data collection, the crystals were transferred directly in the previous solution, with the inclusion of 10–20%(*v*/*v*) glycerol as cryoprotectant. The crystals were mounted in a rayon fibre loop and placed in a boiling nitrogen stream at 100 K.

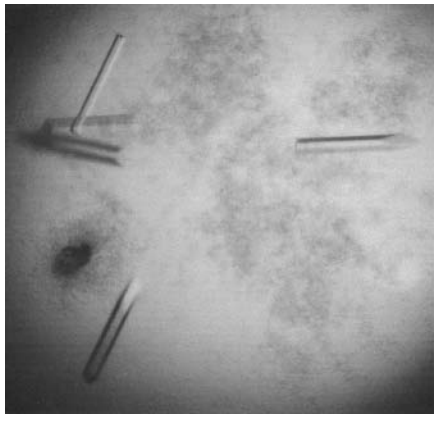

Figure 1
Crystals of *B. subtilis* GlvA. The crystals shown have dimensions of approximately 0.05 × 0.05 × 0.3 mm.

2.2. Data collection and processing

Crystals of native GlvA diffracted only very weakly in the home laboratory. Native X-ray diffraction data were therefore collected from a single crystal flash-frozen at 100 K at the European Synchrotron Radiation Facility (ESRF), beamline ID14-4, using an ADSC QUANTUM 4 charge-coupled device (CCD) detector. 135° of data, to a resolution of 2.2 Å, were collected with an oscillation of 1° and an exposure of 1 s per image. Data were processed and reduced using the *DENZO* and *SCALEPACK* programs (Otwinowski & Minor, 1997).

3. Results and discussion

GlvA crystals grow over a wide pH range from 6.0 to 8.0. The best crystals were obtained at pH 7.0–7.5. Manganese ions are required in the crystallization droplets for optimal diffraction from the resulting crystals. GlvA crystals belong to the centered orthorhombic space group *I*222 (or its enantiomorph $I2_12_12_1$) with unit-cell dimensions $a = 83.26, b = 102.56, c = 145.31$ Å. Synchrotron radiation was essential for data collection, since crystals of GlvA diffract extremely weakly using Cu $K\alpha$ radiation in the home laboratory. Data were collected on beamline ID14-4 at the ESRF and 157330 observations were merged to give 28257 unique reflections, with an $R_{\rm merge}$ ($\sum_{hkl}\sum_i |I_{hkli} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i \langle I_{hkl}\rangle$) of 0.074, a mean $I/\sigma(I)$ of 17.5, a multiplicity of observation of 5.6 and a completeness of 94.5% in the 18–2.2 Å resolution range. 2801 observations were rejected during the data-reduction procedure. In the outer resolution shell (2.28–2.2 Å), the $R_{\rm merge}$ is 0.35 with a mean $I/\sigma(I)$ of 3.7, a multiplicity of observation of 4.7 and a completeness of 94.5%.

GlvA has a molecular weight of 50513 Da. Assuming there is one molecule in the asymmetric unit, the crystal packing density was determined to be 3.04 Å^3 Da^{-1}, corresponding to a solvent content of 59.6% (Matthews, 1968). Native GlvA is known to be active as a tetramer in the presence of Mn^{2+}/NAD(H). This would clearly suggest that the most likely space group is *I*222, with the tetramer being formed by the crystallographic 222 symmetry. Family 4 of the glycoside hydrolases pose a number of mechanistic problems. For example, how do members of this family appear to tolerate both equatorial (β-linked) and axial (α-linked) substrates in the active site, and what are the roles of the divalent metal and NAD(H)? In particular, the chemical mechanism for glycosidic bond cleavage remains unclear. GlvA catalyses the hydrolysis of maltose-6-phosphate to form glucose-6-phosphate and glucose (typical of a true hydrolase) but may possess a different or additional activity (Thompson *et al.*, 1998). We believe that knowledge of the structure of GlvA will permit resolution of these mechanistic conundrums. Heavy metals are currently being screened and selenomethionine protein being produced to aid rapid structure determination.

The authors would like to thank the staff of the ESRF and EMBL Grenoble for provision of data-collection facilities and the EU for funding. The structural biology laboratory at York is supported by the BBSRC. GJD is a Royal Society University Research Fellow.

References

Bouma, C. L., Reizer, J., Reizer, A., Robrish, S. A. & Thompson, J. (1997). *J. Bacteriol.* **179**(13), 4129–4137.
Burstein, C. & Kepes, A. (1971). *Biochim. Biophys. Acta*, **230**, 52–63.
Hengstenberg, W., Kohlbrecher, D., Witt, E., Kruse, R., Christiansen, I., Peters, D., Pogge von Strandmann, R., Stadtler, P., Koch, B. & Kalbitzer, H. R. (1993). *FEMS Microbiol. Rev.* **12**(1–3), 149–163.
Hengstenberg, W., Reiche, B., Eisermann, R., Fischer, R., Kessler, U., Tarrach, A., De Vos, W. M., Kalbitzer, H. R. & Glaser, S. (1989). *FEMS Microbiol. Rev.* **5**(1–2), 35–42.
Henrissat, B. & Bairoch, A. (1993). *Biochem. J.* **293**, 781–788.
Henrissat, B. & Davies, G. J. (1997). *Curr. Opin. Struct. Biol.* **7**(5), 637–644.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Koshland, D. E. (1953). *Biol. Rev.* **28**, 416–436.
Kundig, W., Ghosh, S. & Roseman, S. (1964). *Proc. Natl Acad. Sci. USA*, **52**, 1067–1074.
Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessières, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Cummings, N. J., Daniel, R. A., Denizot, F., Devine, K. M., Düsterhöft, A., Ehrlich, S. D., Emmerson, P. T., Entian, K. D., Errington, J., Fabret, C., Ferrani, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, N., Ghim, S.-Y., Glaser, P., Goffeau, A., Golightly, E. J., Grandi, G., Guiseppi, G., Guy, B. J., Haga, K., Haiech, J., Harwood, C. R., Hénaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M.-F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.-M., Levine, A., Liu, H., Masuda, S., Mauël, C., Médigue, C., Medina, N., Mellado, R. P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.-H., Parro, V., Pohl, T. M., Portetelle, D., Porwollik, S., Prescott, A. M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose., M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S. J., Serror, P., Shin, B.-S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A., Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K.,Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H.-F., Zumstein, E., Yoshikawa, H. & Danchin, A. (1997). *Nature (London)*, **390**(6657), 249–256.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Meadow, N. D., Fox, D. K. & Roseman, S. (1990). *Annu. Rev. Biochem.* **59**, 497–542.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Parker, L. L. & Hall, B. G. (1990). *Genetics*, **124**(3), 455–71.
Postma, P. W., Lengeler, J. W. & Jacobson, G. R. (1993). *Microbiol. Rev.* **57**(3), 543–594.
Reizer, J., Michotey, V., Reizer, A. & Saier, M. H. Jr (1994). *Protein Sci.* **3**(3), 440–450.
Reizer, J., Saier, M. H. Jr, Deutscher, J., Grenier, F., Thompson, J. & Hengstenberg, W. (1988). *Crit. Rev. Microbiol.* **15**(4), 297–338.
Roseman, S. (1989). *FEMS Microbiol. Rev.* **5**(1–2), 3–11.
Saier, M. H. Jr. & Reizer, J. (1992). *J. Bacteriol.* **174**(5), 1433–8.
Sinnott, M. L. (1990). *Chem. Rev.* **90**, 1171–1202.
Thompson, J., Gentry-Weeks, C. R., Nguyen, N. Y., Folk, J. E. & Robrish, S. A. (1995). *J. Bacteriol.* **177**(9), 2505–2512.
Thompson, J., Pikis, A., Ruvinov, S. B., Henrissat, B., Yamamoto, H. & Sekiguchi, J. (1998). *J. Biol. Chem.* **273**(42), 27347–27356.
Wilson, G. & Fox, C. F. (1974). *J. Biol. Chem.* **249**(17), 5586–5598.
Yamamoto, H., Uchiyama, S., Fajar, A. N., Ogasawara, N. & Sekiguchi, J. (1996). *Microbiology*, **142**(6), 1417–1421.