Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Cloning, purification and characterization of the 6-phospho-3-hexulose isomerase YckF from *Bacillus subtilis*

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Received 29 January 2001
Accepted 4 May 2001

The enzyme 6-phospho-3-hexulose isomerase (YckF) from *Bacillus subtilis* has been prepared and crystallized in a form suitable for X-ray crystallographic analysis. Crystals were grown by the hanging-drop method at 291 K using polyethylene glycol 2000 monomethyl-ether as precipitant. They diffract beyond 1.7 Å using an in-house Cu $K\alpha$ source and belong to either space group $P6_522$ or $P6_122$, with unit-cell parameters $a = b = 72.4$, $c = 241.2$ Å, and have two molecules of YckF in the asymmetric unit.

1. Introduction

6-Phospho-3-hexulose isomerase (PHI) catalyses the isomerization of D-*arabino*-3-hexulose-6-phosphate to D-fructose-6-phosphate, as illustrated in Fig. 1. PHI is a key enzyme in the fixation of carbon, in the form of formaldehyde, by methylotrophic bacteria. This process is part of the ribulose monophosphate (RuMP) pathway, the key function of which is the condensation of formaldehyde with ribulose-5-phosphate by 3-hexulose-6-phosphate synthase (HPS) to form D-*arabino*-3-hexulose-6-phosphate, which in turn is isomerized by PHI to fructose-6-phosphate. The subsequent metabolism of fructose-6-phosphate leads to the regeneration of the pentose phosphate acceptor and the net production of triose phosphate (Strøm *et al.*, 1974). Until recently, it was believed that such enzyme systems were restricted solely to methylotrophic organisms. However, the recent explosion of information arising from genome-sequencing projects has led to the identification of similar genes in non-methylotrophs such as *B. subtilis* (Kunst *et al.*, 1997; Reizer *et al.*, 1997; Yasueda *et al.*, 1999). Investigation at the gene-expression level suggests that in this organism these gene products play a specific role in detoxification. For example, when *B. subtilis* was cultured in the presence of formaldehyde, the expression of PHI (*i.e.* YckF) and HPS (*i.e.* YckG) was induced. The observation that methanol, formate and methylamine did not elicit a similar response and the fact that induction of YckF and YckG expression was dependent on another gene, *yckH*, strongly suggest that these three genes function as a specific detoxification system for formaldehyde in this organism (Yasueda *et al.*, 1999).

The *yckF* gene from *B. subtilis* consists of a 558 bp open reading frame (ORF) which encodes a 19.96 kDa protein (Kunst *et al.*, 1997). YckF exhibits significant homology (34 and 37%, respectively) with functional PHIs from the obligate methylotrophic bacterium *Methylomonas aminofaciens* 77a (Sakai *et al.*, 1999) and the facultative methylotrophic bacterium *Mycobacterium gastri* MB19 (Mitsui *et al.*, 2000; Fig. 2). YckF also shares 22.8% identity with the putative CP0226 gene of *Chlamydophila pneumoniae* AR39 and three putative genes from *Haemophilus influenzae*, HI0143 (18%), HI1678 (18.6%) and HI0754 (14.8%). Similarity (16.2%) also exists to a fructose-6-phosphate aminotransferase gene, *glmS*, from *B. subtilis*. Although PHI enzymes have been purified and characterized from a range of organisms (Beardsmore *et al.*, 1982; Ferenci *et al.*, 1974; Kato *et al.*, 1977; Sakai *et al.*, 1999; Mitsui *et al.*, 2000), little is known about their catalytic mechanism. Metal-ion dependency is a common feature amongst previously characterized sugar isomerases, with cobalt, magnesium and manganese predominantly implicated (Watt, 1998). However, the PHI from *Methylococcus capsulatus* has been shown to be fully active in the absence of cations and to be inhibited by various divalent cations (Ferenci *et al.*, 1974). However, it is currently unknown whether YckF is active in the absence of cations. Despite the importance of PHI in terms of carbon fixation, detoxification and its potential in biotransformation applications, little is known about its structure or function. In this paper, we present the cloning and over-expression of the *yckF* gene using an *Escherichia coli* expression system, together with its purification, crystallization and preliminary X-ray diffraction analysis.

2. Experimental

2.1. Cloning, expression and purification

PfuTurbo DNA polymerase (Stratagene) and the oligonucleotide primers 5′-CATAT-

GAAAACGACTGAATACG-3′ and 5′-GGATCCCTATTCAAGGTTTGCGTG-3′ were used to amplify the *yckF* gene from *B. subtilis* 168 (ATCC 23857) genomic DNA. This fragment encoded the *yckF* gene flanked by *Nde*I and *Bam*HI restriction sites. The 567 bp fragment was cloned using the A-tailing procedure for cloning blunt-ended PCR fragments into pGEMT-Easy (Promega). The insert was subcloned into the expression vector pET28a (Novogen) on an *Nde*I/*Bam*HI restriction fragment, generating the plasmid pBSISO1. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3), which had been made competent by the method of Hanahan (1983). The transformants were cultured on LB agar supplemented with 50 µg ml^{-1} kanamycin at 310 K. Cultures for induction were inoculated from a 5 ml 16 h culture and grown in LB supplemented with 50 µg ml^{-1} kanamycin. Isopropyl-thio-β-D-galactoside was added to a final concentration of 1 m*M* when the optical density at 600 nm had reached 0.7, after which incubation was continued for a further 16 h at a reduced temperature of 295 K. The bacteria were harvested by centrifugation, resuspended in 20 m*M* Na_2HPO_4 buffer pH 7.4 containing 0.5 *M* NaCl and 10 m*M* imidazole and sonicated on ice for a total of 1 min using a 10 s on and 10 s off cycle. Cell debris was removed by centrifugation at 30 000*g* for 30 min. The crude extract was loaded onto a PD-10 column (Pharmacia Biotech) containing a 2 ml bed volume of nickel-charged Fast Flow Chelating Sepharose. Purification was achieved as described by the manufacturer, except for the inclusion of two additional washing steps with 100 m*M* imidazole. The eluted protein was buffer exchanged into 20 m*M* Na HEPES buffer pH 7.4 using a Sephadex PD-10 G-25M column (Pharmacia Biotech). The purity of YckF was assessed by SDS–PAGE analysis. The purified protein was concentrated using a 10 kDa molecular-weight cutoff centrifugation membrane (Amicon).

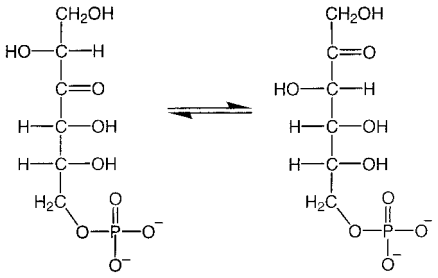

Figure 1
6-Phospho-3-hexulose isomerase catalyses the isomerization of D-*arabino*-3-hexulose-6-phosphate to D-fructose-6-phosphate.

2.2. PHI assay

To determine whether recombinant YckF was active, it was assayed according to the method of Arfman *et al.* (1990) with modifications as described by Yasueda *et al.* (1999). This multi-enzyme linked assay features ribose-5-phosphate conversion to ribulose-5-phosphate by ribose phosphate isomerase. The product of this first reaction is then combined with formaldehyde by 3-hexulose-6-phosphate synthase, generating D-*arabino*-3-hexulose-6-phosphate, the substrate for YckF. The product of YckF, fructose-6-phosphate, then undergoes isomerization by phosphoglucose isomerase, yielding glucose-6-phosphate, which is finally converted to 6-phosphoglucono-δ-lactone by glucose-6-phosphate dehydrogenase, leading to the production of NADPH, which is followed at 340 nm.

2.3. Crystallization

Crystals of YckF were grown by vapour-phase diffusion using the hanging-drop method in Falcon 3047 multiwell plates at 291 K. The drops comprised an equal volume, 1 or 2 µl, of YckF at a concentration of 20 mg ml^{-1} mixed with reservoir solution. Crystals of YckF appeared in numerous conditions of the crystallization screen as described by Brzozowski & Walton (2001) after only a few hours. These initial conditions were optimized by the addition of 10 or 20%(*v*/*v*) of either glycerol, 2-methyl-2,4-pentandiol (MPD) or ethylene glycol and reducing the protein concentration to 10 mg ml^{-1}. When the reservoir solution comprised 0.8 *M* sodium formate, 1 m*M* EDTA, 25%(*w*/*v*) polyethylene glycol 2000 monomethylether and 10%(*v*/*v*) MPD in 20 m*M* Na HEPES buffer pH 7.4, hexagonal crystals appeared after 24 h and had approximate dimensions of 0.2 × 0.2 × 0.1 mm (Fig. 3). This buffer solution was also a cryoprotectant, enabling crystals to be mounted directly into a rayon-fibre loop and placed into a stream of N_2 gas at 120 K.

2.4. Data collection and processing

Native X-ray diffraction data were collected in the home laboratory from a single crystal at 120 K using a 345 mm MAR Research imaging-plate detector on a Rigaku rotating-anode RU-200 X-ray generator with a Cu target operating at 50 kV and 100 mA with the use of focusing X-ray optics (MSC). 40.4° of data to a resolution of 2.6 Å were collected with an oscillation range of 0.2° and an exposure time of 10 min per image. Data were processed and reduced using the *DENZO* and *SCALEPACK* programs (Otwinowski & Minor, 1997).

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B. subtilis          ----------------MKTTEYVAEILNELHNSAAYISNEEADQLADHILSSHQIFTAGAG  45
M. gastri            MTQAAEADGAVKVVGDDITNNLSLVRDEVADTAAKVDPEQVAVLARQIVQPGRVFVAGAG  60
M. aminofaciens      ----------------MNKYQELVVSKLTNVINNTAEGYDDKILSLVDAAGRTFIGGAG  43
                                     .:    : .:: :            :   :  . : * .***

B. subtilis          RSGLMAKSFAMRLMHMGFNAHIVGEILTPPLAEGDLVIIGSGSGETKSLIHTAAKAKSLH 105
M. gastri            RSGLVLRMAAMRLMHFGLTVHVAGDTTTPAISAGDLLLVASGSGTTSGVVKSAETAKKAG 120
M. aminofaciens      RSLLVSRFFAMRLVHAGYQVSMVGEVVTPSIQAGDLFIVISGSGSTETLMPLVKKAKSQG 103
                     ** *: :  ****:* *  . :.*:  **.:  ***.:: **** *. ::  . .**.

B. subtilis          GIVAALTINPESSIGKQADLIIRMPGSPKDQSNGSYKTIQPMGSLFEQTLLLFYDAVILK 165
M. gastri            ARIAAFTTNPDSPLAGLADAVVIIPAAQKT-DHGSHISRQYAGSLFEQVLFVVTEAVFQS 179
M. aminofaciens      AKIIVISMKAQSPMAELADLVVPVGGNDANAFDKTHG--MPMGTIFELSTLWFLEATIAK 161
                     . : .:: :.:*.:.  ** :: : .      . ::      *::**   : . :*.: .

B. subtilis          LME-----------------KKGLDSETMFTHHANLE                        185
M. gastri            LWD-----------------HTEVEAEELWTRHANLE                        199
M. aminofaciens      LVDQKGLTEEGMRAIHANLEHTEVEAEELWTRHANLE                         198
                     * :                  :. :::* ::*:*****
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Figure 2
A *ClustalW* (1.8) multiple sequence alignment of 6-phospho-3-hexulose isomerases from *B. subtilis*, *Mycobacterium gastri* and *Methylmonas aminofaciens*. The position of identical residues (asterisks) and conserved (:) and semi-conserved (.) substitutions are indicated.

3. Results and discussion

Purified YckF was apparently pure as judged by SDS–PAGE analysis and ran as a single well defined band when conducting non-denaturing polyacrylamide gel electrophoresis (data not shown). MALDI-TOF mass-spectrometric analysis indicated that recombinant YckF had a molecular mass of 22.16 kDa, consistent with the predicted value, 22.13 kDa, which includes the N-terminal polyhistidine tag. The purified protein was very stable and could be stored at 277 K at a protein concentration of 20 mg ml^{-1} in 20 m*M* Na HEPES buffer pH 7.4. To determine whether YckF was active, it was assayed, as described in §2.2, by measuring the production of NADPH at

340 nm. Recombinant YckF had a specific activity of 193 µmol of $NADP^+$ reduced per minute per milligram of pure enzyme. Crystals of YckF belong to space group $P6_522$ or $P6_122$, with unit-cell parameters $a = b = 72.4$, $c = 241.2$ Å, and most likely have two protein molecules in the asymmetric unit. Crystals of YckF diffracted very strongly in the home laboratory, but the resolution of such data was constrained to 2.6 Å owing to the long c axis. 202 840 observations were merged to give 12 384 unique reflections, with an $R_{\rm merge}$ $(\sum_{hkl}\sum_i |I_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_i I_{hkl})$ of 0.019, a mean $I/\sigma(I)$ of 48.9, a multiplicity of observation of 4.2 and a completeness of 94% in the 12–2.6 Å resolution range. The data were very strong, with an $R_{\rm merge}$ of 0.027 and a mean $I/\sigma(I)$ of 32.4 and a multiplicity of observation of 4.4 in the outer resolution shell (2.69–2.6 Å). Assuming that there are two molecules in the asymmetric unit, the crystal packing density was determined to be 2.07 $Å^3\,Da^{-1}$, which corresponds to a solvent content of 40.2% (Matthews, 1968). Alternatively, though less likely, one molecule in the asymmetric unit corresponds to a density and solvent content of 4.15 $Å^3\,Da^{-1}$ and 70.1%, respectively.

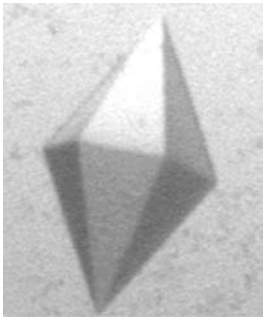

Figure 3
A single crystal of YckF from *B. subtilis*. This hexagonal crystal with dimensions 0.1 × 0.1 × 0.2 mm was obtained using 20 m*M* Na HEPES buffer pH 7.4 containing 0.8 *M* sodium formate, 1 m*M* EDTA, 25%(*w*/*v*) polyethylene glycol 2000 monomethylether and 10%(*v*/*v*) MPD.

The structure of YckF will allow illumination of the catalytic mechanism and substrate specificity of this important family of proteins and will give an insight into the closely related homologues of unknown function from various pathogenic bacteria such as *H. influenzae* and *C. pneumoniae*. These putative enzymes, although distantly related, appear to possess the same catalytic machinery as YckF from *B. subtilis*. Structural data will also allow the rational design of catalysts for specific biotransformations using PHI with other RuMP pathway enzymes. Selenomethionine-substituted protein is currently being produced to aid rapid structure determination.

The authors would like to thank the BBSRC Wellcome Trust and EU for financial assistance. GJD is a Royal Society University Research Fellow. Collaboration between the Universities of York and Sunderland was assisted by the provision of a BBSRC Structural Biology Centre award to the York Structural Biology Laboratory.

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