Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray crystallographic studies of recombinant thermoresistant gluconate kinase GntK from *Escherichia coli*

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Received 12 March 2001
Accepted 29 May 2001

The thermoresistant gluconate kinase GntK from *Escherichia coli*, an essential enzyme in gluconate metabolism, has been expressed, purified and crystallized. For crystallization, the hanging-drop vapour-diffusion method was used with polyethylene glycol (PEG) 6000 and lithium chloride as precipitants. Three crystal forms belonging to the monoclinic space group $C2$ or the orthorhombic space groups $P2_12_12_1$ and $P2_12_12$ were obtained. The unit-cell parameters are $a = 75.0$, $b = 79.3$, $c = 70.2$ Å, $\beta = 105.3^\circ$ ($C2$), $a = 52.0$, $b = 79.3$, $c = 89.8$ Å ($P2_12_12_1$) and $a = 70.1$, $b = 74.1$, $c = 78.9$ Å ($P2_12_12$). In all three crystal forms, there are two molecules in the asymmetric unit; the different forms occur in the same crystallization drop. The crystals diffract to at least 2.0 Å using synchrotron radiation.

1. Introduction

In *E. coli*, gluconate can be provided as an exogenous carbon source or can also be produced by the direct oxidation of glucose (Hommes *et al.*, 1984; van Schie *et al.*, 1985). In order to serve as a growth substrate for *E. coli*, gluconate must first be transported into the cell and phosphorylated to gluconate-6-phosphate by gluconate kinase (Cohen, 1951), which is the first step of gluconate metabolism before entering degradative pathways. The major flow of carbon from gluconate is *via* the Entner–Doudoroff pathway, with a minor flow of carbon through the pentose phosphate pathway (Eisenberg & Dobrogosz, 1967). The enzymes of the Entner–Doudoroff pathway, the gluconate transport system and gluconate kinase are specifically induced by gluconate (for reviews, see Conway, 1992; Peekhaus & Conway, 1998). The transport of gluconate into the cells and the initial phosphorylation is performed by two different systems, GntI and GntII, respectively (Bächi & Kornberg, 1975). The predominant system, GntI, contains a high- and a low-affinity gluconate transporter and a thermoresistant gluconate kinase GntK (Izu *et al.*, 1997). The GntII system contains, among other proteins, a thermosensitive gluconate kinase IdnK (formerly named GntV). The presence of two different systems has led to much confusion and the GntII system was thought to be a subsidiary system for gluconate uptake and catabolism (Isturiz & Celaya, 1997). However, it has recently been shown that the genes of the GntII system encode a novel metabolic pathway for catabolism of L-idonate in which D-gluconate is an intermediate. The normal inducer is apparently L-idonate, but D-gluconate can, under certain conditions, induce the GntII pathway (Bausch *et al.*, 1998). The finding that *E. coli* can utilize the Entner–Doudoroff pathway for oxidative glucose metabolism and the widespread distribution of this pathway in microorganisms suggests that this pathway is of greater importance in nature than earlier considered (Fraenkel, 1987).

GntK functions as a homodimer with a subunit molecular mass of 19.5 kDa comprising 175 amino acids. The enzyme amino-acid sequence contains an ATP-binding motif similar to those found in other sugar kinases, G*XXXX*GK[TS] (Tong *et al.*, 1996). The optimum temperature for GntK activity is 318 K and it has been shown that 100% of the activity was retained after incubation at 303 K for 30 min. Under the same conditions, the thermosensitive enzyme IdnK was reported to lose more than 75% of its activity (Izu *et al.*, 1996). The GntK sequence does not show significant overall sequence identity to any known sugar kinase except IdnK, for which no structural data are available. Comparison studies of the GntK and IdnK sequences of *E. coli* showed them to be 45% identical (Tong *et al.*, 1996). Here, we describe crystallization and preliminary X-ray analysis of the thermoresistant gluconate kinase from *E. coli.*

2. Materials and methods

2.1. Cloning and expression of GntK

The *gntK* gene of *E. coli* K-12 was amplified by PCR using chromosomal DNA of strain W3110 (prototrophic wild type) as template. PCR primers GntK*Eco*RI, 5′-CC**GAATTC**-

TTGTATTGTGGGGGCAC-3′, and GntK-*Bam*HI, 5′-CC**GGATCC**GTTAATGTAGT-CACTACTTA-3′, were utilized to introduce engineered unique restriction sites for *Eco*RI and *Bam*HI, respectively (in bold). The DNA was purified by agarose-gel electrophoresis and the resulting 0.6 kbp DNA fragment was isolated with a QiaEx kit (Qiagen, Hilden, Germany), restricted with *Eco*RI plus *Bam*HI and ligated to the vector pBM20 (Boehringer Mannheim, Germany), which had been cleaved with the same enzymes, resulting in pBM20*gntK* (Krämer, 2000). Subcloning of the 0.6 kbp *Eco*RI + *Bam*HI fragment was performed by ligation to the vector pJF119EH (Fürste *et al.*, 1986), which had likewise been cleaved with *Eco*RI plus *Bam*HI, and by selection for ampicillin-resistant transformants. For cloning and IPTG-controlled expression, strain DH5α (Hanahan, 1983) was used throughout. Shaking-flask cultures of recombinant cells were grown at 310 K in LB media with 100 mg l^{-1} of ampicillin to an optical density of 0.5 at OD 600 nm before induction with IPTG (100 µ*M* final concentration) for 4 h. Cells of the late-exponential growth phase were harvested by centrifugation and washed with 50 m*M* ice-cold Tris–HCl buffer pH 8.0 containing 3 m*M* $MgCl_2$ and 1 m*M* DTT.

2.2. Purification and enzyme assay

Cells were lysed by ultrasonic treatment in a UP 200S sonifier at an amplitude of 70% for 6 × 30 s with cooling in an ethanol–ice bath and 30 s cooling intervals. Cell debris was spun down for 1 h at 277 K at 30 000*g* and the supernatants were used as the cell-free extract. Cell-free extracts were concentrated using a 100 kDa cutoff ultrafiltration membrane (Amicon); owing to its relatively small size of ∼40 000 kDa, gluconate kinase was thus separated from the majority of other proteins, which were retained. Following two anion-exchange column chromatography steps (Q-Sepharose FF; Pharmacia, Freiburg, Germany and fractogel DEAE tentacle, Merck, Darmstadt, Germany) and gel filtration, gluconate kinase was almost homogeneous (>95% as judged by Coomassie staining of SDS–PAGE gels), with a specific activity of about 25 U per mg of protein, where U is the amount of enzyme which will catalyse the transformation of 1 µmol of substrate per minute. The protocol of Izu *et al.* (1996), in which phosphogluconate dehydrogenase (Boehringer Mannheim) is utilized as auxiliary enzyme, was employed for assaying gluconate kinase activity. The appearance of the reduced form of NADP was followed spectrophotometrically in a Shimadzu UV160A photometer at an OD of 340 nm in a thermostated (298 K) cuvette with a 1 cm light path.

Protein purity was assessed using polyacrylamide gel stained with Coomassie R 350 prior to crystallization.

Table 1
Crystallographic data and data-collection statistics.

Values for the highest resolution shell are in parentheses.

Space group	*C*2	$P2_12_12_1$	$P2_12_12$
Resolution (Å)	2.1 (2.14–2.10)	2.0 (2.03–2.00)	2.33 (2.37–2.33)
Mosaicity (°)	0.5	0.45	1.2
No. of observations	76408	135276	58100
No. of unique reflections	22646	25758	14913
Redundancy	3.4	5.3	3.9
R_{sym} (%)	6.0 (19.2)	5.4 (27.1)	12.7 (29.3)
Data completeness (%)	97.9 (76.1)	99.8 (98.4)	81.3 (31.3)
$I/\sigma(I)$	19.6 (3.9)	12.6 (4.4)	9.1 (2.4)

2.3. Crystallization

The concentration of gluconate kinase used for crystallization was 10 mg ml^{-1} in the purification buffer, 20 m*M* Tris–HCl pH 8.0, 3 m*M* $MgCl_2$ and 1 m*M* DTT. Initial crystallization trials were carried out at 293 and 277 K with Hampton Research Crystal Screen kits using the hanging-drop vapour-diffusion method. Within a few days, small crystals were obtained in the PEG/LiCl screen with 20%(*w*/*v*) PEG 6000, 1 *M* LiCl pH 6 and with 30%(*w*/*v*) PEG 6000, 1 *M* LiCl pH 6–8 at 277 K from 2 µl protein solution mixed with 2 µl of reservoir solution. After optimization of the crystallization conditions, crystals useful for crystallographic structure determination (Fig. 1) were obtained with a reservoir solution containing 17–20%(*w*/*v*) PEG 6000, 1.4–1.7 *M* LiCl and 0.1 *M* pH 6.0 MES with a well volume of 0.7 ml.

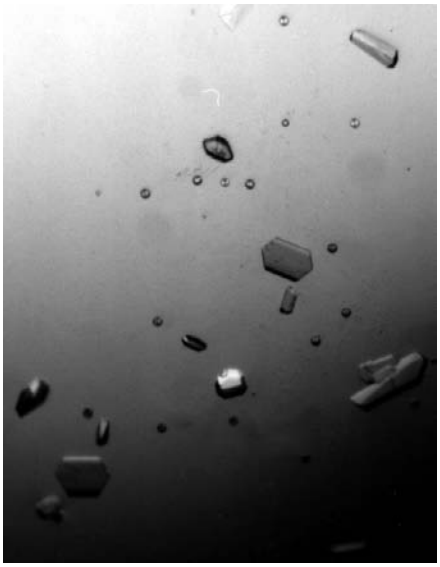

Figure 1
Different crystal forms of thermoresistant gluconate kinase are present in the same drop. The size of the largest crystal is approximately 0.15 × 0.10 × 0.05 mm.

2.4. Data collection and processing

For data collection, the crystals were directly flash-frozen in nitrogen gas at 100 K (Oxford Cryosystems Cryostream Cooler); no additional cryoprotectant was necessary. Data were collected at beamline 711, MAX-Laboratory, Lund, Sweden using a MAR 345 image plate with an oscillation angle of 1° at wavelengths and crystal-to-detector distances of 1.282 Å and 210 mm (*C*2), 0.995 Å and 270 mm ($P2_12_12_1$), and 1.104 Å and 290 mm ($P2_12_12$), respectively. The data were indexed, processed and scaled using the *HKL* package (Otwinowski & Minor, 1997). Space-group determination was performed using the autoindexing option in *DENZO* (Otwinowski & Minor, 1997) and analysis of the pseudo-precession images generated using the program *PATTERN* (Lu, 1999). The crystals belong to space groups *C*2, $P2_12_12_1$ and $P2_12_12$, with unit-cell parameters a = 75.0, b = 79.3, c = 70.2 Å, β = 105.3° (*C*2), a = 52.0, b = 79.3, c = 89.8 Å ($P2_12_12_1$) and a = 70.1, b = 74.1, c = 78.9 Å ($P2_12_12$). Crystals of different space groups may be present in the same crystallization drop and cannot be distinguished by their morphology. Crystallographic data were collected to 2.1 Å (*C*2), 2.0 Å ($P2_12_12_1$) and 2.3 Å ($P2_12_12$). Complete data-collection statistics are shown in Table 1. The Matthews coefficient (V_M) and the corresponding solvent content assuming two molecules in the asymmetric unit are 2.6 $Å^3\ Da^{-1}$ and 51.9% (*C*2), 2.4 $Å^3\ Da^{-1}$ and 47.7% ($P2_12_12_1$) and 2.6 $Å^3\ Da^{-1}$ and 52.7% ($P2_12_12$), respectively (Matthews, 1968). The native Patterson maps clearly show a high peak in both of the orthorhombic space groups, indicating non-crystallographic symmetry. The self-rotation functions only show peaks representing

crystallographic symmetry, indicating that non-crystallographic twofold axes, if present, must be parallel to twofold crystallographic axes.

3. Conclusions

We have obtained three different crystal forms of the thermoresistant gluconate kinase which diffract to 2 Å resolution. The V_M of the unit cells and the native Patterson maps suggest two molecules in the asymmetric unit in all cases. The use of MIR methods to solve the structure have so far failed owing to the non-isomorphism of the derivatized crystals, which show varying unit-cell dimensions. We have, therefore, expressed a selenomethionine-substituted enzyme in order to be able to use the MAD method.

We gratefully acknowledge the MAX-Laboratory, Lund, Sweden for provision of synchrotron radiation. We thank Marco Krämer for clone pBM20*gntK*, Ulli Degner for expert technical assistance and Hermann Sahm for his continuous support. This work was supported by the Swedish Foundation for Strategic Research (SSF) through the Structural Biology Network (SBNet).

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