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Crystallization and preliminary X-ray characterization of queD from *Bacillus subtilis*, an enzyme involved in queuosine biosynthesis

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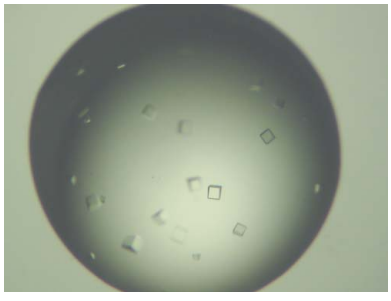
QueD (previously named ykvK) is one of several enzymes involved in the biosynthesis of the hypermodified nucleoside queuosine. Queuosine is incorporated into tRNA at position 34 of four tRNAs: $tRNA^{His}$, $tRNA^{Asp}$, $tRNA^{Asn}$ and $tRNA^{Tyr}$. The crystallization and preliminary X-ray crystallographic studies of queD are described here. The recombinant protein from *Bacillus subtilis* was overproduced in *Escherichia coli* and crystallized using the hanging-drop vapor-diffusion method from 25% PEG 600, 100 m*M* NaCl and sodium citrate buffer pH 5.5 at 291 K. The crystals diffract to 3.6 Å resolution and belong to the cubic space group $F4_132$, with unit-cell parameter $a = 240.88$ Å.

1. Introduction

To date, 107 modified nucleosides that occur in nucleic acids have been identified. Most of the modified nucleosides described thus far have been found in tRNA (Limbach *et al.*, 1994). These modified nucleosides vary in complexity, ranging from the simple to the more complex. One of the most complex modifications found in tRNA is queuosine (Q). It has been shown that the function of Q is to modulate translation by affecting the flexibility of the anticodon loop, which results in higher fidelity during translation (Morris *et al.*, 1999). Queuosine is located in the wobble position (position 34) of four tRNAs: $tRNA^{His}$, $tRNA^{Asp}$, $tRNA^{Asn}$ and $tRNA^{Tyr}$, in prokaryotes and eukaryotes (Slany & Kersten, 1994). The only known exceptions are *Saccharomyces cerevisiae*, *Thermus thermophilus* and *Mycoplasma* sp. (Morris & Elliott, 2001). In contrast to prokaryotes and eukaryotes, archaea contain a hypermodified nucleoside structurally similar to queuosine termed archaeosine (Iwata-Reuyl, 2003). Only prokaryotes can synthesize Q *de novo*, while eukaryotic organisms rely on Q produced by intestinal flora (Kuchino *et al.*, 1976). Even though this modification is conserved throughout prokaryotes and eukaryotes, Q is not essential for life. However, it has been observed that *queD*-knockout mutants of some *Shigella* strains lose their pathogenicity (Durand *et al.*, 2000), whereas in eukaryotes germ-free mice lacking Q showed severely diminished reproductive potential (Farkas, 1980; Reyniers *et al.*, 1981). Moreover, it has been reported that the absence of Q modification has a negative effect on cell proliferation (Lin *et al.*, 1980) and has been implicated in the development of some types of tumors in humans (Baranowski *et al.*, 1994).

The formation of Q is a multistep reaction that can be divided into three stages (Fig. 1). The first stage is the biosynthesis of $preQ_1$ from GTP. The second stage is the incorporation of $preQ_1$ into tRNA; a detailed mechanism has been proposed for this stage (Xie *et al.*, 2003). In the third stage, $preQ_1$, having been incorporated into tRNA, undergoes further modification that yields the final product, Q. Even though GTP was identified as the precursor of queuosine over 30 years ago (Kuchino *et al.*, 1976), the enzymes responsible for carrying out the biosynthesis of $preQ_1$ from GTP could not be identified using classical genetic approaches owing to the fact that the absence of Q is not associated with any obvious phenotype (Noguchi *et al.*, 1982). However, the large number of sequenced genomes and increasing number of computational tools have made it possible to predict the functions of genes based on clustering (de Crécy-Lagard,



2007). Recent bioinformatics searches revealed four new *Bacillus subtilis* enzymes: queC, queD, queE and queF (previously named ykvJ, ykvK, ykvL and ykvM; Reader *et al.*, 2004). Together with the newly discovered GTP cyclohydrolase I (El Yacoubi *et al.*, 2006), these four enzymes are implicated in the biosynthesis of $preQ_1$ from GTP. Of these four enzymes, only queF, which is an NADPH-dependent oxidoreductase that reduces the nitrile group in $preQ_0$ to amino group in $preQ_1$, has been characterized to date (Van Lanen *et al.*, 2005).

2. Methods and results

2.1. Gene cloning

The *queD* gene was PCR-amplified from *B. subtilis* genomic DNA using forward primer 5′-TAGGGCGAGCTCAAGGAGATAT-ACATATGCATAAATTGTTATCTCAAATTTATCCGCA-3′ and reverse primer 5′-TAGGCAAAGCTTTCAGCCATTCAAACCA-CCCTTTTTCG-3′, which contained *Sac*I and *Hin*dIII restriction sites, respectively. The gene was cloned into pLM-1 vector and the results were confirmed by agarose gel electrophoresis and sequencing.

2.2. Protein expression and purification

The pLM-1 vector carrying untagged recombinant queD was transformed into the *Escherichia coli* BL21 (DE3) cell line. The cells were grown at 310 K in 2 l LB medium supplemented with 50 µg ml^{-1} ampicillin until the OD_{600} reached 0.6. At this point the cells were induced with 1 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG). After 12 h, the cells were harvested and lysed using a French press. The lysis buffer contained 100 m*M* Tris–HCl pH 8.5, 20 m*M* NaCl, 1 m*M* DTT (DEAE-A buffer). The cell lysate was centrifuged and the supernatant was passed through a preparatory DEAE anion-exchange column. The protein was eluted with DEAE-B buffer (100 m*M* Tris–HCl pH 8.5, 1 *M* NaCl, 1 m*M* DTT). Fractions containing queD were pooled and concentrated using an Amicon centrifugal filter device from Millipore. The buffer was exchanged to HiLoad-A buffer (100 m*M* Tris–HCl pH 7.0, 1.5 *M* ammonium sulfate, 1 m*M* DTT) and loaded onto a HiLoad hydrophobic column.

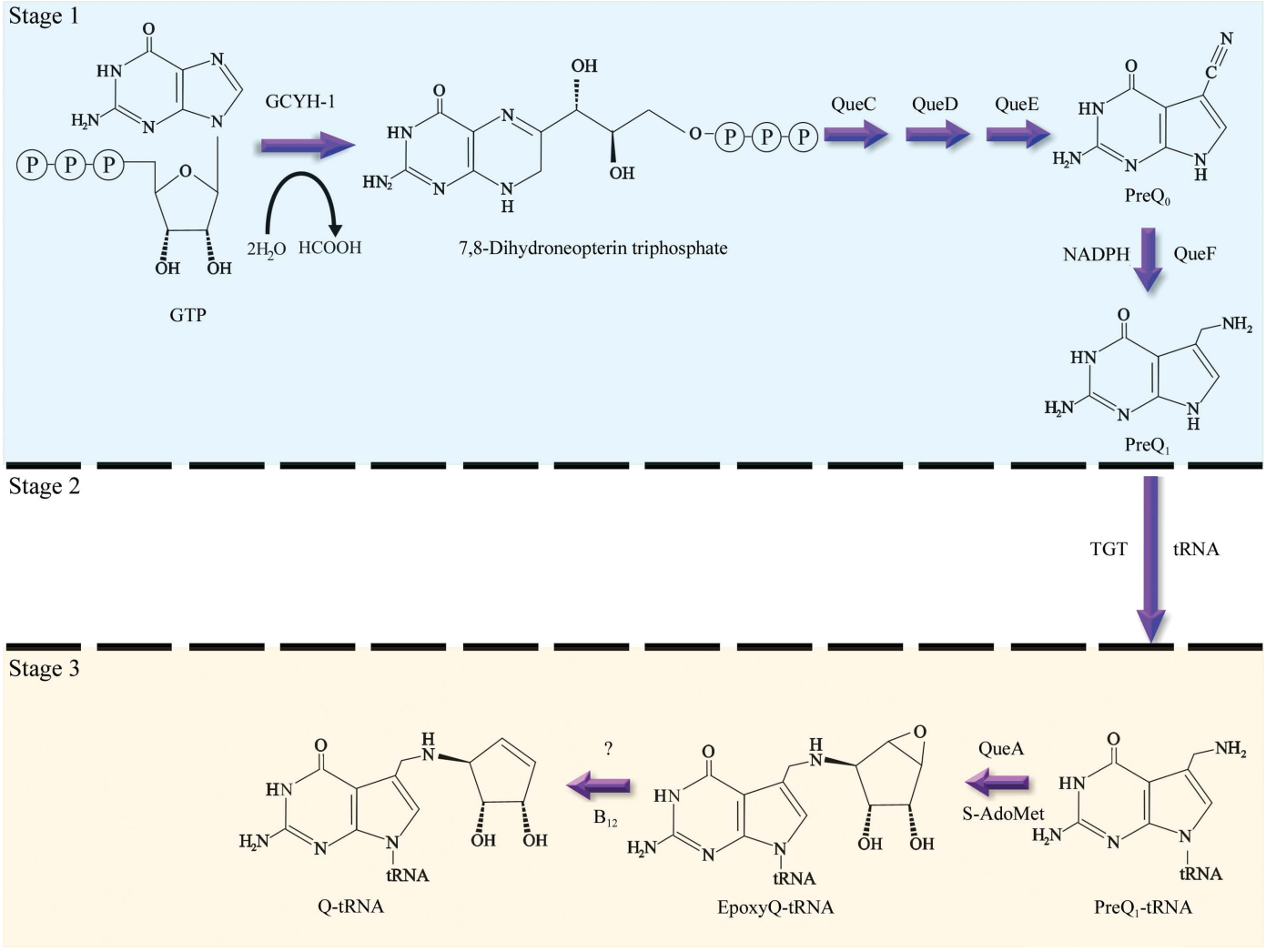


Figure 1
The biosynthetic and RNA-modification steps involved in the formation of queuosine (Q). In the biosynthesis stage (stage 1), GTP is hydrolyzed to 7,8-dihydroneopterin triphosphate by GTP cyclohydrolase I. queC, queD and queE catalyze an unknown set of reactions generating $preQ_0$, which is reduced to $preQ_1$ by queF. In the transfer stage (stage 2), $preQ_1$ is incorporated into tRNA by the enzyme tRNA-guanine transglycosylase (TGT). Finally, in the RNA-modification stage (stage 3), $preQ_1$ is converted to epoxyQ by queA and then an unidentified enzyme reduces epoxyQ to Q.

The protein was eluted from the column with HiLoad-B buffer (100 m*M* Tris–HCl pH 7.0, 1 m*M* DTT). The queD fractions were concentrated, buffer-exchanged back to DEAE-A buffer and loaded onto a high-resolution ion-exchange MonoQ column. A gradient from DEAE-A to DEAE-B buffer was used to elute the protein from the MonoQ column. In the last step, queD was further purified on a Superdex 200 size-exclusion column. The elution volume of queD on Superdex 200 corresponds to a protein of approximately 65 kDa in size, indicating that queD is a tetramer in solution (data not shown).

2.3. Crystallization

Purified queD at a concentration of 10 mg ml^{-1} was subjected to a grid crystallization screen using the vapor-diffusion method. The crystallization screen was composed of the following precipitants: 10–60% ammonium sulfate, 10–60% PEG 600 in combination with 100 m*M* NaCl, 10–35% PEG 6000 in combination with 100 m*M* NaCl and 10–35% PEG 6000 in combination with 1 *M* NaCl. All crystallization combinations were tested at four pH values (5.5, 6.0, 7.0 and 8.0). The crystallization trials were performed at two temperatures: 291 and 277 K. The total number of conditions was 192. Hanging drops made up of 0.8 µl protein solution and 0.8 µl precipitant solution were set up in 24-well plates (Hampton Research, USA). Cubic crystals were obtained in 25% PEG 600, 100 m*M* NaCl and 100 m*M* sodium citrate buffer pH 5.5 at 291 K (Fig. 2). The crystals were cryoprotected in a solution identical to the crystallization solution but with the addition of 30% glycerol.

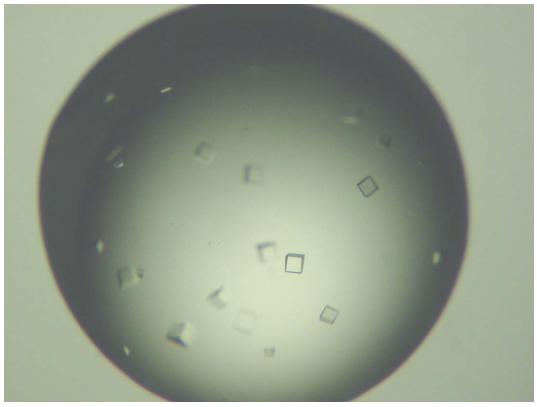


Figure 2
Crystals of queD grown in 25% PEG 600, 100 m*M* NaCl and 100 m*M* sodium citrate buffer pH 5.5 at 291 K using the hanging-drop vapor-diffusion technique. The approximate dimensions of the crystals are 0.05 × 0.05 × 0.05 mm.

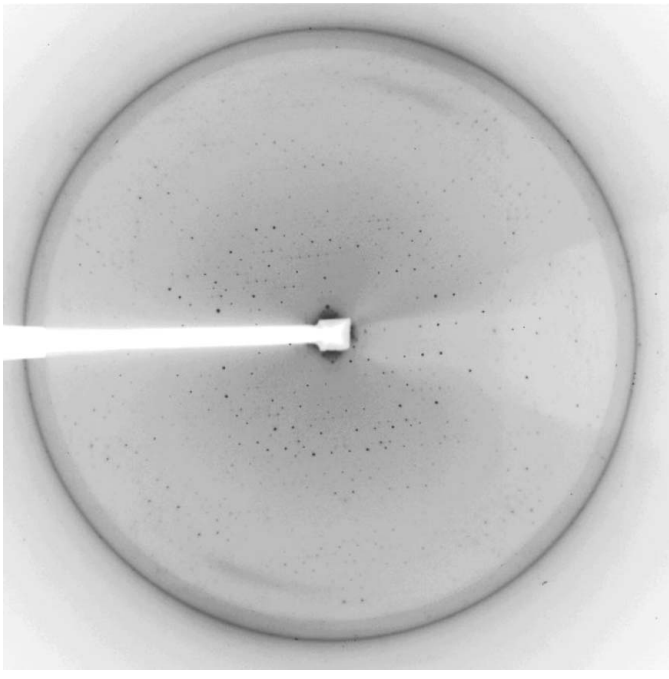


Figure 3
A 1.0° oscillation X-ray image of queD. The image was taken at the 22-ID beamline at the Advanced Photon Source (APS), Argonne, Illinois, USA.

Table 1
X-ray data-collection parameters.

Values in parentheses are for the highest resolution shell.

Space group	$F4_132$
Unit-cell parameter (Å)	a = 240.88
Resolution range	50.0–3.6
No. of measured reflections	160964
No. of unique reflections	7366
Completeness (%)	100.0 (100.0)
$R_{\rm merge}$	6.3 (25.9)
$\langle I/\sigma(I)\rangle$	6.6 (2.1)
Matthews coefficient (Å^3 Da^{-1})	5.58
Solvent content (%)	43.17
No. of molecules in ASU	4

2.4. Crystallographic data collection and processing

The crystals were screened for diffraction quality using an in-house X-ray source and an R-AXIS IV^{++} imaging-plate area detector (Rigaku, Japan). A 3.6 Å data set was collected from a flash-frozen cubic crystal at the 22-ID beamline at the Advanced Photon Source (APS), Argonne, Illinois, USA (Fig. 3). Data collection was performed with a 1° oscillation angle for each diffraction image and a total oscillation range of 100°. The exposure time for each image was 5 s. Processing and scaling were accomplished using *HKL*-2000 (Otwinowski & Minor, 1997). The crystals belong to space group $F4_132$, with unit-cell parameter a = 240.88 Å (Table 1). If four protein molecules per asymmetric unit are assumed, the Matthews coefficient is 2.16 Å^3 Da^{-1}. This value corresponds to a solvent content of 43.17% (Matthews, 1968). Further improvements of the crystals aimed towards achieving improved resolution are in progress.

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