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Expression, purification, crystallization and preliminary X-ray analysis of wild-type and of an active-site mutant of glyceraldehyde-3-phosphate dehydrogenase from *Campylobacter jejuni*

David S. Tourigny, Paul R. Elliott, Louise J. Edgell, Gregg M. Hudson and Peter C. E. Moody*

Henry Wellcome Laboratories for Structural Biology, Henry Wellcome Building, University of Leicester, Leicester LE1 9HN, England

Correspondence e-mail: peter.moody@leicester.ac.uk

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The genome of the enteric pathogen *Campylobacter jejuni* encodes a single glyceraldehyde-3-phosphate dehydrogenase that can utilize either $NADP^+$ or NAD^+ as coenzymes for the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. Here, the cloning, expression, purification, crystallization and preliminary X-ray analysis of both the wild type and an active-site mutant of the enzyme are presented. Preliminary X-ray analysis revealed that in both cases the crystals diffracted to beyond 1.9 Å resolution. The space group is shown to be $I4_122$, with unit-cell parameters a = 90.75, b = 90.75, c = 225.48 Å, α = 90.46, β = 90.46, γ = 222.79°; each asymmetric unit contains only one subunit of the tetrameric enzyme.

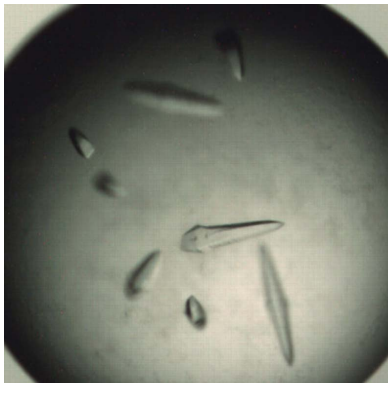
1. Introduction

Campylobacter jejuni is a foodborne human gastrointestinal pathogen that is carried as a commensal organism in avian species. It is estimated that approximately 1% of the European human population are infected by *Campylobacter* spp. each year (World Health Organization, 1994) and *C. jejuni* is known to cause a spectrum of diseases upon invasion of the intestinal mucosa. These can include chronic enteritis and bloody diarrhoea, with rare occurrences of extra-intestinal infiltration of the peripheral nervous system, resulting in Guillain–Barré and Miller–Fisher syndromes (Nachamkin *et al.*, 1998). Doctors in the public health system observe milder cases of *C. jejuni* infection more than any other known cause of foodborne disease in England and Wales (Adak *et al.*, 2002); therefore, this species poses a serious health and economic problem to the human population.

The full genomic sequence of *C. jejuni* strain NCTC11168 has been reported, enabling post-genomic analysis to identify homologues of the genes that play essential roles in many cellular metabolic processes. *C. jejuni* appears to maintain a full set of gluconeogenic enzymes whilst lacking several of those thought to be essential for glycolysis (Parkhill *et al.*, 2000). Furthermore, the genes for carbohydrate transporters appear to be lacking and this is consistent with growth experiments, which show a requirement for amino acids but not for glucose (Velayudhan *et al.*, 2004). Together, these observations suggest that amino acids and not carbohydrates are the primary substrates for *C. jejuni* metabolism.

The *C. jejuni* genome appears to encode only a single copy of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (*cgap*), unlike many bacteria, including the closely related *Helicobacter pylori* (Baltrus *et al.*, 2009), which have two GAPDH homologues.

The GAPDH substrate glyceraldehyde 3-phosphate (G3P) can be an intermediate in both the catabolism of sugars (glycolysis) and the reverse anabolic pathway (gluconeogenesis) in bacteria and eukaryotes and plays a key role in photosynthesis. This glycolytic/gluconeogenic intermediate is also able to feed into the pentose-phosphate shunt for the generation of $NADP^+$ and three-carbon



sugars for nucleotide biosynthesis. Furthermore, G3P can be generated through the Entner–Doudoroff pathway, which is an alternative for glucose oxidation (Entner & Doudoroff, 1952). The absence of a complete set of glycolytic enzymes and therefore the lack of an apparent glycolytic role for *C. jejuni* GAPDH (cGAPDH) may have allowed significant evolutionary divergence from the previously studied homologous enzymes that retain the glycolytic function and thus cGAPDH is worth structural and biochemical investigation.

In this study, we have expressed, purified and crystallized the GAPDH of *C. jejuni* that is encoded by the *cgap* gene (gi:218563007, NCBI, NIH). Kinetic analysis, which will be presented elsewhere, has demonstrated that this enzyme is able to utilize both $NADP^+$ and NAD^+ in solution, a property thought to be unique to plants and archaea (Falini *et al.*, 2003). In order to observe analogues of the Michaelis complex in homologous enzymes, several groups have mutated the active-site cysteine, thus preventing acylenzyme formation (*e.g.* Didierjean *et al.*, 2003; Mukherjee *et al.*, 2010). We have also prepared and analyzed crystals of a C150S cGAPDH mutant with this aim; preliminary biochemical data has shown that, as expected, the mutant is unable to turn over G3P in the presence of either NAD^+ or $NADP^+$. Obtaining structures of this mutant with G3P bound will provide insight into the ternary (Michaelis) complex of this enzyme.

2. Materials and methods

2.1. Cloning and overexpression

The full *cgap* nucleotide sequence (gi:218563007, NCBI, NIH) was cloned from *C. jejuni* NCTC11168 genomic DNA into pET151/D (Invitrogen) containing an N-terminal His_6 tag linked by a TEV protease site. The primer sequences for the forward and reverse amplification of the *cgap* gene were CACCATGGCTGTAAAAG-TTGCTATAAATGG and GAGGGTACCTTATTTGCAATATAT-ACTGC, respectively. Dideoxy sequencing confirmed the full-length *cgap* sequence. *Escherichia coli* strain Rosetta DE3 transformed with pET151/D-*cgap* was grown to an OD_{600} of 0.7 at 303 K in 2YT medium supplemented with 60 µg ml^{-1} ampicillin and 35 µg ml^{-1} chloramphenicol. Cells were induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 200 µ*M* and were incubated for 12 h overnight at 291 K prior to harvesting.

2.2. Purification

Cell pellets were resuspended in lysis buffer (20 m*M* Na_2HPO_4, 500 m*M* NaCl, 20 m*M* imidazole pH 7.4) supplemented with protease-inhibitor cocktail VII (Calbiochem). The suspension was

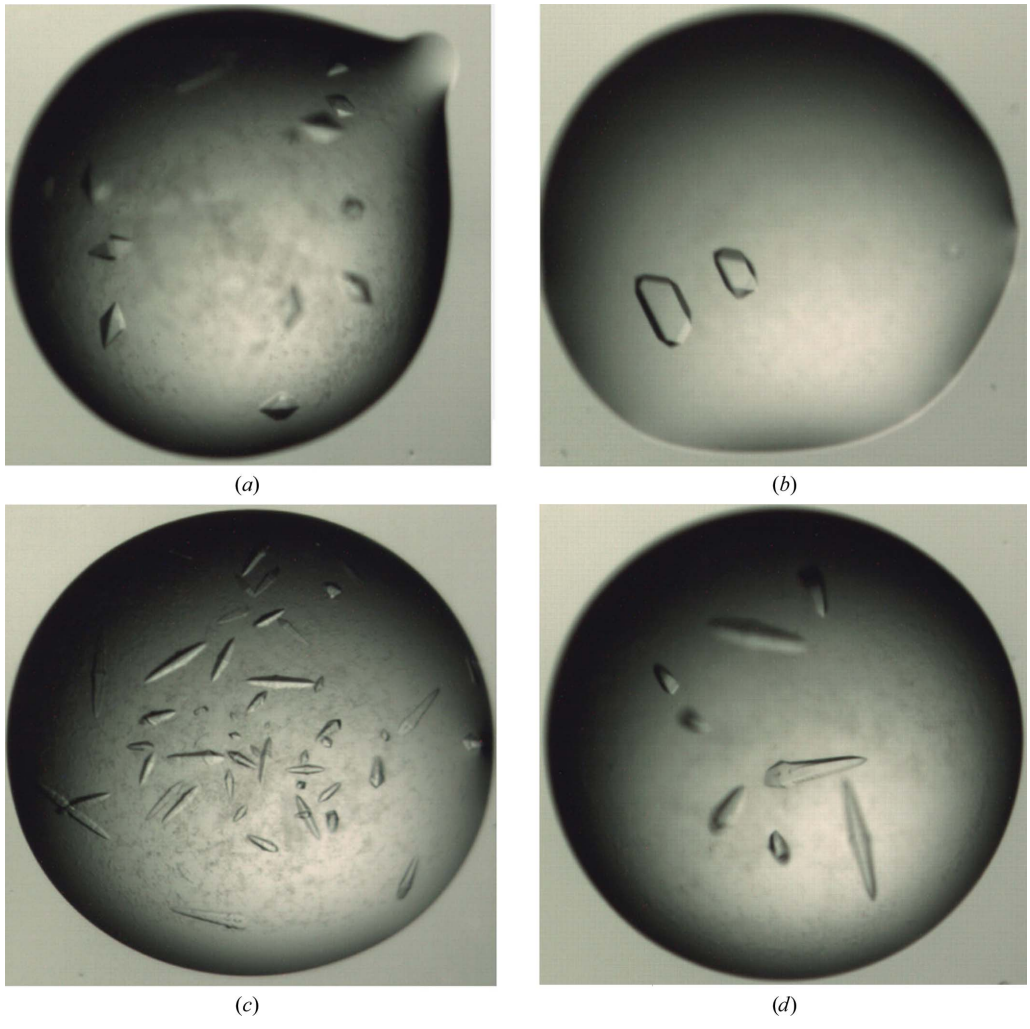


Figure 1
Crystals of wild-type cGAPDH grown in 1 m*M* $NADP^+$ using the Omza 1K and 4K Salt Screens (Emerald BioSystems). Crystals grew in 500 nl sitting drops with 20%(*w*/*v*) PEG 4K (*a*–*c*) or PEG 1K (*d*) as precipitant and various salts added to a final concentration of 200 m*M*: ammonium fluoride (*a*), magnesium acetate (*b*), sodium tartrate (*c*) and sodium/potassium tartrate (*d*). Crystals from (*d*) were used in data collection.

Table 1
Summary of diffraction data statistics.

Values in parentheses are for the highest resolution shell.

	cGAPDH + NADP$^+$	cGAPDH C150S
Wavelength (Å)	0.931	0.9795
Space group	$I4_122$	$I4_122$
Unit-cell parameters (Å, °)	$a = b = 90.75$, $c = 225.48$	$a = b = 90.46$, $c = 222.79$
Resolution limits (Å)	45.4–1.9 (2.0–1.9)	63.96–1.91 (1.91–1.96)
No. of observations	268069 (38889)	257750 (12978)
No. of unique observations	37590 (5379)	36172 (2626)
Completeness	100.0 (100.0)	100 (99.9)
$\langle I/\sigma(I)\rangle$	12.9 (4.2)	14.9 (2.4)
$R_{\rm merge}$†	0.13 (0.43)	0.102 (0.572)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\langle I(hkl)\rangle$ is the mean intensity of this reflection.

sonicated at 12 kHz for 5 s (five cycles) and insoluble cell debris was removed by high-speed centrifugation. The supernatant was passed through a 0.2 µm filter and loaded onto a 5 ml Hi-Trap Nickel Sepharose (Amersham Biosciences) column pre-equilibrated with lysis buffer; His_6-cGAPDH was then eluted with a linear gradient of elution buffer (20 m*M* Na_2HPO_4, 500 m*M* NaCl, 500 m*M* imidazole pH 7.4). Fractions containing recombinant cGAPDH were pooled (a total of 3 ml) and His-tagged TEV protease (Invitrogen) was added in accordance to the manufacturer's instructions before 12 h dialysis against a 2 l volume of dialysis buffer (20 m*M* Na_2HPO_4, 50 m*M* NaCl, 1 m*M* DTT pH 7.2) at 277 K. The sample was then reapplied onto a 5 ml Hi-Trap Nickel Sepharose column to remove TEV protease and uncleaved protein. The eluate was collected and judged to be >99% pure by SDS–PAGE analysis. cGAPDH was concentrated to 11 mg ml^{-1} using an Amicon Ultra-15 centrifugal filter unit (10 kDa molecular-weight cutoff; Millipore) and buffer-exchanged into 20 m*M* Tris–HCl, 100 m*M* NaCl, 1 m*M* DTT pH 7.2 prior to crystallization.

2.3. Site-directed mutagenesis

The C150S active-site mutant was generated *via* a PCR-based approach using the QuikChange Site-Directed Mutagenesis II kit (Stratagene) on the pET151/D-*cgap* plasmid. The primers GAAAGCATTATTTCTAATGCAAGTAGTACAACAAATTG (forward) and CAGGACCTAAACAATTTGTTGTACTACTTGCATTAG (reverse) were designed in accordance with the company's recommendations. PCR reaction products were analyzed by agarose-gel electrophoresis. Dideoxy sequencing confirmed that the mutagenesis reaction had been successful and the cGAPDH C150S mutant was expressed and purified using a protocol equivalent to that used for the wild-type cGAPDH protein.

2.4. Crystallization and data collection

Crystal-growth conditions for wild-type and mutant cGAPDH [amino acids 1–332, with an additional N-terminal tetrapeptide (Ser-Pro-Phe-Thr) from the hexahistidine tag and TEV site; 36.6 kDa] were screened by the sitting-drop vapour-diffusion technique using the Wizard I and II and Cryo I and II crystallization screening kits (Emerald BioSciences). 100 nl protein solution (with 1 m*M* NADP$^+$ added to the wild-type cGAPDH) and 100 nl precipitant were dispensed with a Genomics Solutions Cartesian Honeybee 8+1 (Harvard Bioscience) onto 96-well MRC plates (Innovadyne) with reservoirs containing 80 µl precipitant in a humidified chamber. Drops were monitored for crystal growth in CrystalProHT (TriTek) plate-storage and imaging systems at 277 and 293 K. Initial hits in several drops gave thin plates and these conditions typically contained 20%(*w*/*v*) PEG 1K as precipitant. The first round of refinement focused on pH and PEG concentration, but yielded no improvement in crystal thickness. This was followed by a second round of crystal-quality optimization in 500 nl sitting drops with an 80 µl reservoir volume using the Omza 1K and 4K Salt Screens (Emerald BioSystems) and larger crystals were found in conditions containing formate, acetate or tartrate as anions (Fig. 1). cGAPDH C150S was found to crystallize in the absence of added NADP$^+$ under similar conditions but with PEG 1K and sodium/potassium tartrate and the largest crystals were found in 2 µl sitting drops (with 500 µl reservoir volumes) containing 25%(*w*/*v*) PEG 1K, 175 m*M* sodium/potassium tartrate and 100 m*M* sodium acetate pH 4.5. Suitable wild-type and mutant crystals of diffraction quality grew after 48 h at 277 K and were prepared for cryocrystallography by transferring them into a mixture consisting of the crystallization condition with glycerol incorporated to a final concentration of 20%(*v*/*v*). The crystals were then vitrified in a stream of boiled-off liquid nitrogen at 100 K. Diffraction data for wild-type cGAPDH were collected on the ID14-3 beamline at the ESRF, Grenoble at a fixed wavelength of 0.931 Å using an ADSC Q4R CCD detector; a typical diffraction pattern is shown in Fig. 2. Data from the C150S mutant crystals were collected on the I02 beamline at the Diamond Light Source, Harwell, England. Intensities were measured using *MOSFLM* (Leslie, 1992), with the autoindexing routines for both proteins giving a solution consistent with a tetragonal *I*-centred cell. The data were scaled using *SCALA* (Evans, 2006) and this was consistent with Laue symmetry 4/*mmm*; thus, the only possible space groups are $I422$ or $I4_122$ and successful molecular replacement using chain *O* of PDB entry 1gd1 (the glyceraldehyde-3-phosphate dehydrogenase of *Bacillus stearothermophilus*, Skarzyński *et al.*, 1987; 51% identity) in *Phaser* (Read, 2001) was only obtained for space group $I4_122$. This gives a calculated Matthews coefficient ($V_{\rm M}$; Matthews, 1968) of 2.79 Å^3 Da^{-1} and 55%

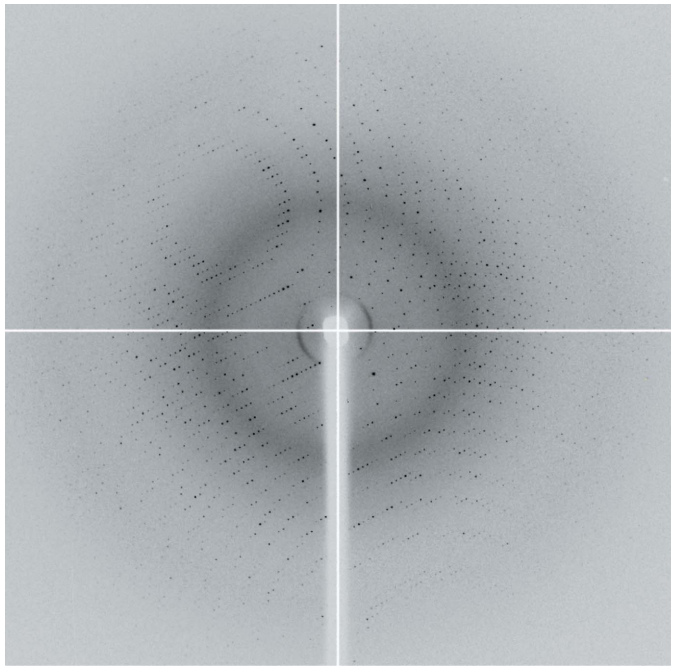


Figure 2
Typical diffraction image of a wild-type cGAPDH crystal grown in the presence of NADP$^+$. Images were collected from a single cryocooled crystal with a crystal-to-detector distance of 172.27 mm using a wavelength of 0.931 Å. The edges of the detector show a $d_{\rm min}$ of 1.88 Å.

solvent content if the asymmetric unit consists of a single GAPDH monomer; to our knowledge, this is the first time such an arrangement has been reported for a tetrameric GAPDH. Data-collection statistics are given in Table 1.

3. Results and discussion

Wild-type cGAPDH has been cloned, expressed, purified and crystallized in the presence of $NADP^+$. In addition, isomorphous crystals were obtained of a C150S active-site mutant in the absence of cofactor. In both cases crystals diffracted to 1.9 Å resolution and this will enable high-resolution structures to be determined and the observation of any conformational changes induced upon coenzyme binding. The structure of the enzyme bound to NAD^+ is being pursued in order to understand the molecular mechanism of the ability of the enzyme to use either NAD^+ or $NADP^+$ and to compare this with homologous enzymes with specific coenzyme requirements. Work is also in progress to produce a ternary complex with glyceraldehyde 3-phosphate for structural analysis.

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