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Preliminary crystallographic studies of an extremely thermostable KDG aldolase from *Sulfolobus solfataricus*

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Crystals have been grown of 2-keto-3-deoxygluconate aldolase (KDG aldolase) from the hyperthermophilic archaeon *Sulfolobus solfataricus* that diffract to 2.2 Å resolution. The enzyme catalyses the reversible aldol cleavage of 2-keto-3-dexoygluconate to pyruvate and glyceraldehyde, the third step of a modified non-phosphorylated Entner–Doudoroff pathway of glucose oxidation. *S. solfataricus* grows optimally at 353 K and the enzyme itself has a half-life of 2.5 h at 373 K. Knowledge of the crystal structure of KDG aldolase will further understanding of the basis of protein hyperthermostability and create a target for site-directed mutagenesis of active-site residues, with the aim of altering substrate specificity. Three crystal forms have been obtained: orthorhombic crystals of space group $P2_12_12_1$, which diffract to beyond 2.15 Å, monoclinic crystals of space group $C2$, which diffract to 2.2 Å, and cubic crystals of space group $P4_232$, which diffract to 3.4 Å.

1. Introduction

S. solfataricus is a thermoacidophilic archaeon that grows optimally at 353–358 K and pH 2–4 (Zillig *et al.*, 1980). The organism utilizes a modified version of the Entner–Doudoroff pathway of glucose oxidation in which there is no net yield of ATP and most of the intermediates are not phosphorylated (De Rosa *et al.*, 1984). The same modified pathway has been found in the archaeon *Thermoplasma acidophilium* (Budgen & Danson, 1986). The pathway in *S. solfataricus* begins with the NAD(P)-dependent dehydrogenation of glucose to gluconate and is followed by dehydration to form KDG (Giardina *et al.*, 1986). The enzyme 2-keto-3-deoxygluconate aldolase (KDG aldolase) then catalyses the reversible aldol cleavage of 2-keto-3-deoxygluconate (KDG) to pyruvate and glyceraldehyde (Fig. 1). The ability to synthesize C—C bonds (the reverse of the aldol cleavage reaction), combined with its extreme thermostability and the use of non-phosphorylated substrates, makes KDG aldolase an attractive candidate for industrial exploitation.

KDG aldolase is composed of four identical monomers of molecular weight (MW) 33 kDa. The amino-acid sequence shows 28% identity with *N*-acetylneuraminate lyase (NAL) and 29% identity with dihydrodipicolinate synthase (DHDPS), both from *Escherichia coli* (Buchanan *et al.*, 1999). The three-dimensional crystal structures of these proteins have been solved and show them to possess structural similarities reflecting their 25% sequence identity with each other. The structures are tetramers with 222 point-group symmetry, each monomer being made up of a core α_8/β_8 barrel and a C-terminal α-helical domain. The active site of each monomer is positioned at the centre of the barrel and contains a conserved lysine residue that is involved in Schiff-base formation with the substrates (Izard *et al.*, 1994; Mirwaldt *et al.*, 1995). This active-site lysine and other residues believed to be important to the reaction mechanism in NAL and DHDPS are also conserved in the KDG aldolase amino-acid sequence. It is therefore believed that KDG aldolase is a putative member of the *N*-acetylneuraminate lyase superfamily of Schiff-base-forming aldolases, dehydratases and decarboxylases (Babbitt & Gerlt, 1997; Lawrence *et al.*, 1997).

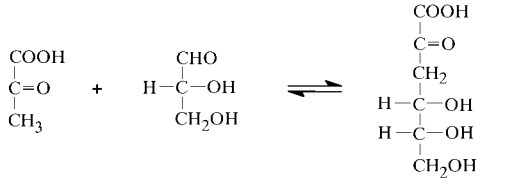

Figure 1
The reaction catalysed by 2-keto-3-deoxygluconate aldolase.

A related enzyme, 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, catalyses the cleavage of KDPG to pyruvate and glyceraldehyde-3-phosphate. The structure of the enzyme from *Pseudomonas putida*, solved to 2.8 Å resolution (Mavridis *et al.*, 1982), shows the enzyme to be a trimer of α_8/β_8 barrels. Comparison of the structure of KDPG aldolase with that of KDG aldolase would allow analysis of the influence of the phosphate group on substrate specificity and enzyme quaternary structure.

Knowledge of the structure of KDG aldolase to atomic resolution would allow site-directed mutagenesis to be performed in order to probe the reaction mechanism and to alter substrate specificity. The three-dimensional structure of KDG aldolase from *S. solfataricus* will also help to further the understanding of hyperthermostability by comparison with the mesophilic enzymes of the NAL superfamily and will contribute to knowledge that may allow thermostable traits to be engineered into other less heat-stable enzymes.

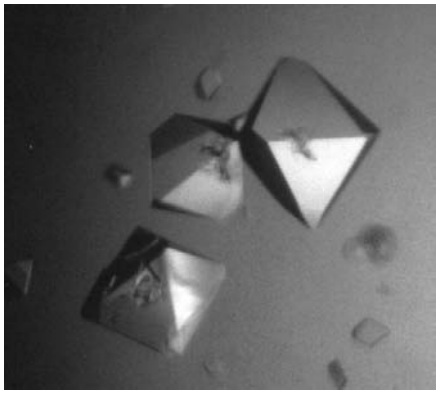

Figure 2
A picture of the orthorhombic crystals of KDG aldolase, which are 200 µm in the longest dimension.

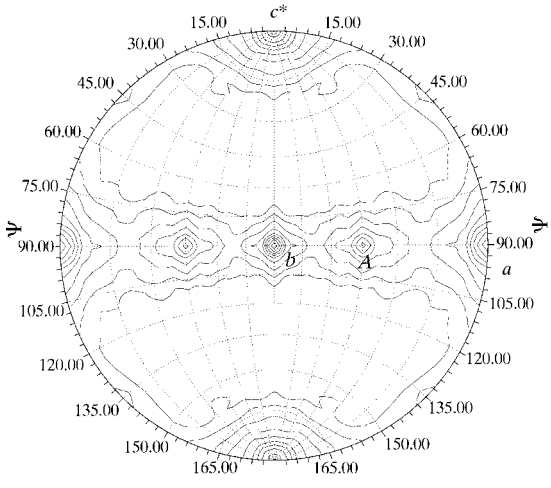

Figure 3
The $\kappa = 180^\circ$ section of the self-rotation function for the orthorhombic crystal form using data from 8 to 4 Å resolution and a sphere of 20 Å. Spherical polar angles are defined as φ, the angle from the Cartesian x axis (a) in the xy plane; ψ, the angle from the z axis (c^*); κ, the rotation around the axis defined by $\varphi\psi$. The peak labelled A at $\varphi = 45^\circ$ is 75% of the height of the origin and results from a non-crystallographic twofold on the ab plane.

Table 1
Data-collection statistics.

Figures in parentheses refer to the highest resolution shells.

	Orthorhombic	Monoclinic	Cubic
Space group	$P2_12_12_1$	$C2$	$P4_232$
Unit-cell parameters (Å,°)	$a = 135.1$, $b = 135.9$, $c = 188.7$	$a = 46.4$, $b = 76.2$, $c = 71.5$, $\beta = 98.1$	$a = b = c = 192.8$
No. measured reflections	344853 (27986)	129781 (12485)	358747 (30810)
No. unique reflections	186606 (18156)	12361 (1217)	16986 (1647)
Resolution (Å)	2.15	2.2	3.4
$R_{\rm merge}$† (%)	6.4 (11.1)	3.9 (14.8)	19.1 (54.3)
Completeness (%)	99.0 (97.4)	98.1 (98.3)	97.5 (97.8)
$I/\sigma(I)$	31.8 (5.7)	27.4 (6.0)	12.6 (3.2)
Outer shell (Å)	2.23–2.15	2.28–2.20	3.52–3.40

† $R_{\rm merge} = \sum|I(k) - \langle I\rangle|/\sum I(k)$, where $I(k)$ is the value of the kth measurement of the intensity of a reflection, $\langle I\rangle$ is the mean value of the intensity of that reflection and the summation is over all measurements.

2. Expression and purification

The KDG aldolase gene from *S. solfataricus* was cloned into the pREC7 expression vector, transformed into *E. coli* JM109 cells and the recombinant protein expressed and purified as described in Buchanan *et al.* (1999). The active protein was eluted from the final anion-exchange column in 20 m*M* Tris–HCl pH 8.5 containing approximately 0.2 *M* NaCl and was then concentrated to 12 mg ml^{-1}.

3. Crystallization and data collection

Crystallization trials were set up by the hanging-drop diffusion method in Linbro plates using Hampton Research Crystal Screen kits (Hampton Research, California, USA). KDG aldolase was concentrated to 12 mg ml^{-1} in 20 m*M* Tris pH 8.5, 0.2 *M* NaCl. Orthorhombic and monoclinic crystals grew in 0.1 *M* citrate pH 4.0, 1.6 *M* ammonium sulfate and had similar tetragonal bipyramidal morphology. A picture of the orthorhombic crystals is shown in Fig. 2. The orthorhombic crystals grew within 1 d, whereas the monoclinic crystals took three months to appear. Crystals of cubic morphology grew in 0.1 *M* citrate pH 4.5, 10%(*v*/*v*) PEG 4000. The monoclinic, orthorhombic and cubic crystals were about 200 µm in length in all directions. The orthorhombic data were collected at the DESY synchrotron, Hamburg, Germany, on beamline BW7B ($\lambda = 0.8345$ Å) at 100 K using 28%(*v*/*v*) glycerol as a cryoprotectant, with a 0.5° oscillation angle. The cubic and monoclinic data were collected at DESY on beamline X31 ($\lambda = 1.04$ Å), with an oscillation angle of 0.5° for the cubic crystal and on beamline X11 ($\lambda = 0.909$ Å) with a 1.5° oscillation angle for the monoclinic crystal. Both data sets were collected at 100 K using 25 and 20%(*v*/*v*) glycerol, respectively, as a cryoprotectant. Data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and the data-collection statistics for the orthorhombic, monoclinic and cubic crystals are shown in Table 1.

Assuming a MW of 33 kDa for each monomer, the orthorhombic and cubic crystal forms are likely to contain two and one tetramers per asymmetric unit, respectively, with solvent contents of 61 and 53% (Matthews, 1968). The monoclinic form is predicted to contain one monomer in the asymmetric unit, with a solvent content of 35%.

4. Analysis of the orthorhombic and monoclinic crystals

The orthorhombic crystals, despite their tetragonal bipyrimidal appearance and the similar lengths of the a and b axes, are clearly orthorhombic, as merging high-resolution reflections in the $P4$ crystal class leads to an $R_{\rm merge}$ of 48.6%. The self-rotation function calculated using *GLRF* (Tong & Rossmann, 1997; Fig. 3) shows a peak 75% of the height of the origin that indicates a possible non-crystallographic twofold on the ab plane at 45° to a. This would suggest that the two tetramers in the asymmetric unit are in the same orientation,

with one of the molecular dyads parallel to *c*. A native Patterson, using data from 20 to 2.16 Å resolution, reveals a peak 42% of the height of the origin at (0.0, 0.04, 0.5) which is consistent with a dyad parallel to *c*, but could also be interpreted as the two tetramers being related by pseudo-centering. Pseudo-centering would lead to systematic weak or absent reflections along 00*l*, which were observed, and together with the *h*00 and 0*k*0 systematic absences suggest the space group $P2_12_12_1$.

Solvent-content calculations predict that the *C*2 monoclinic crystals contain a monomer in the asymmetric unit, despite the tetrameric nature of the protein in solution. We suggest that the length of time taken to grow the crystals (three months) may have led to a high salt concentration in the crystallization drop which favoured dissociation of the tetramers into monomers or dimers. In the latter case, the dimer would be formed by the crystallographic twofold axis. This is consistent with the observation that the interfaces between subunits in thermophilic proteins tend to be stabilized by salt bonds that would be disrupted by an increase in ionic strength (Russell & Taylor, 1995).

We are attempting to solve the structure by molecular replacement using diffraction data from the orthorhombic form with NAL and DHDPS as phasing models and by selenomethionine labelling for multi-wavelength anomalous dispersion phasing.

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