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Crystallization and preliminary crystallographic analysis of NAD$^+$-preferring aldohexose dehydrogenase from the thermoacidophilic archaeon *Thermoplasma acidophilum*

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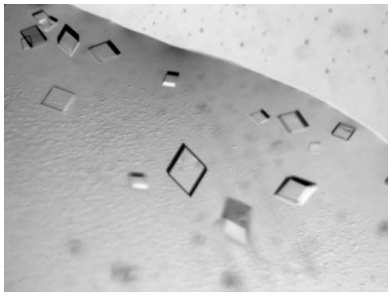
The aldohexose dehydrogenase from the thermoacidophilic archaeon *Thermoplasma acidophilum* (AldT) is a 28 kDa molecular-weight enzyme that catalyzes the oxidation of various aldohexoses, with a preference for NAD$^+$ rather than NADP$^+$ as a cofactor. The recombinant AldT was crystallized using the hanging-drop vapour-diffusion technique at 293 K under several acidic conditions with polyethylene glycol (PEG) and ammonium sulfate as precipitants. Optimization of the initial crystallizations conditions yielded single crystals in solution containing 0.1 *M* sodium acetate pH 4.6, 18%(*w*/*v*) PEG 4000, 0.2 *M* ammonium sulfate and 15%(*v*/*v*) glycerol. An X-ray diffraction data set was collected to a resolution of 2.8 Å.

1. Introduction

NAD(P)$^+$-dependent glucose dehydrogenase (GlcDH; EC 1.1.1.47) is an enzyme that catalyzes the oxidation of β-D-glucose to yield D-glucono-1,5-lactone with concomitant reduction of NAD(P)$^+$ to NAD(P)H. NAD(P)$^+$-dependent GlcDHs are widely distributed in prokaryotic organisms and are classified into two distinct groups. The archaeal GlcDH is a member of the first group, the medium-chain dehydrogenase/reductase family, with a typical molecular weight of 40 kDa. Enzymes of this family are known to contain one or two zinc ions per subunit; one zinc ion plays a catalytic role in the vicinity of the active site, while the second is a structural feature located far from the active site (Jornvall *et al.*, 1987). The archaeal GlcDH was found to participate in the non-phosphorylating variant of the Entner–Doudoroff pathway for pyruvate synthesis (De Rosa *et al.*, 1984; Lamble *et al.*, 2003) that can metabolize both glucose and galactose. Therefore, archaeal GlcDH displays promiscuous specificity for both glucose and galactose (Lamble *et al.*, 2003; Angelov *et al.*, 2005). To date, the structure of GlcDH from the thermoacidophilic archaeon *Thermoplasma acidophilum* (TaGlcDH) has been determined in a substrate-free form (John *et al.*, 1994). In eubacteria, species of the order *Bacillus* possess a GlcDH that belongs to the short-chain dehydrogenase/reductase (SDR) family, with a typical molecular weight of 30 kDa (Jornvall *et al.*, 1995). In contrast to the metabolic function of archaeal GlcDHs, the *Bacillus* GlcDHs are known to also be involved in spore germination (Otani *et al.*, 1986). The crystal structure of *B. megaterium* GlcDH (BmGlcDH) in complex with NAD$^+$ has been determined (Yamamoto *et al.*, 2001). Although the sequence identity between archaeal and eubacterial GlcDHs is less than 15%, these two enzymes are structurally related owing to the presence of the Rossmann fold (a common core motif for nucleotide binding; Rossmann *et al.*, 1974) and both form tetrameric assemblies.

Interestingly, recent genome projects have revealed that *T. acidophilum* contains three ORFs (Ta0754, Ta0191 and Ta0747) with a significant sequence similarity to *Bacillus* GlcDH enzymes. Recently, we have shown that the Ta0754 protein can catalyze the oxidation of various aldohexoses such as D-glucose and 2-deoxy-D-glucose. Moreover, it exhibits remarkable activity toward D-mannose and prefers NAD$^+$ rather than NADP$^+$ as cofactor (Nishiya *et al.*, 2004). The Ta0754 protein, designated here as AldT, is composed of 255 amino-acid residues with a molecular weight of



28 kDa. Our previous size-exclusion chromatographic analysis indicated that the AldT forms a tetramer, as do most enzymes of the SDR family (Nishiya *et al.*, 2004). To date, AldT is the only enzyme known to display an efficient NAD^+-dependent dehydration activity of D-mannose. Although the crystal structure of BmGlcDH in complex with NAD^+ has been reported, no structural information regarding the monosaccharide-recognition mechanism of GlcDHs of the SDR family is available. To elucidate the structural basis of the broad substrate recognition of AldT and its high activity towards D-mannose, we have undertaken a crystallographic study of this enzyme. The D-mannose dehydrogenase activity may be useful for the quantitative determination of D-mannose in clinical tests. The crystallographic analysis of AldT will provide a template for further structure–function correlations by site-directed mutagenesis. Here, we present the crystallization and preliminary X-ray diffraction analysis of AldT.

2. Materials and methods

2.1. Expression and purification of recombinant AldT

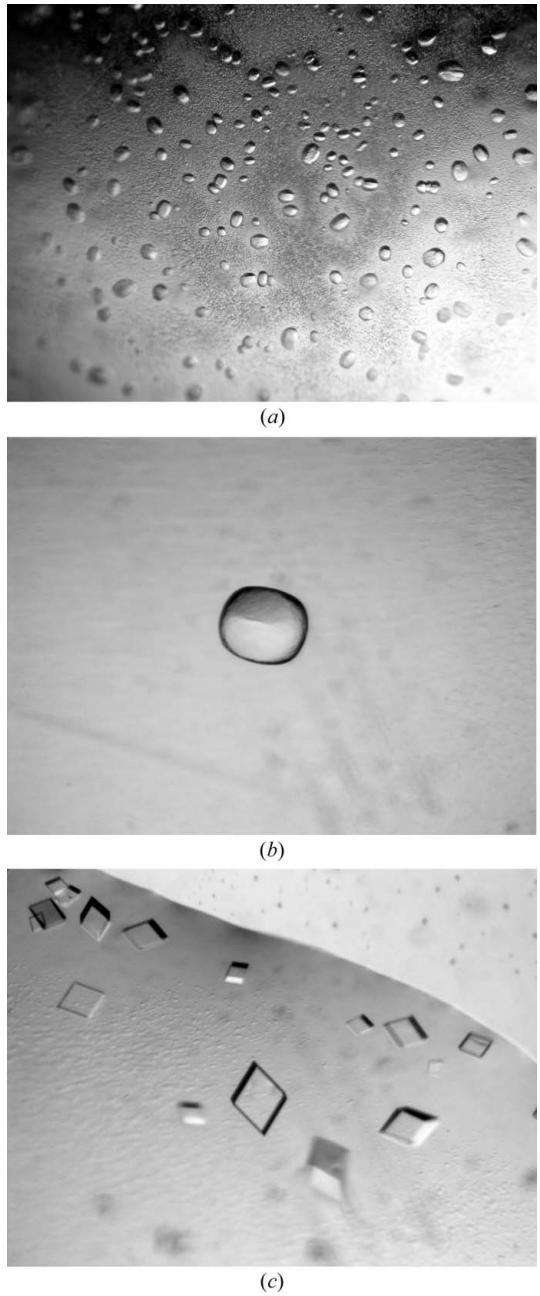
The gene encoding AldT (Ta0754) was amplified by polymerase chain reaction (PCR) and the PCR fragments were digested with *Nco*I and *Xho*I and cloned into the equivalent site of pET28a expression vector (Novagen). The resulting plasmid encoded for full-length AldT and a Leu-Glu sequence before the hexahistidine tag at the C-terminus. The plasmid was transformed into *Escherichia coli* BL21(DE3)-RIL cells. The overexpressed enzyme was purified using Ni–NTA resin (Qiagen) according to the manufacturer's instructions. The purified enzyme was dialyzed against 25 m*M* Tris–HCl buffer pH 7.5 containing 20%(*v*/*v*) glycerol and was frozen at 193 K until use for crystallization screening. The overexpression and purification procedures have been described in detail previously (Nishiya *et al.*, 2004).

2.2. Crystallization

The stock sample was gently thawed on ice and was diluted with 20 m*M* Tris–HCl buffer pH 7.5 and concentrated several times in a centrifugal ultrafiltration device with a 30 kDa cutoff membrane (Millipore) for removal of glycerol. The sample was finally adjusted to approximately 10.0 mg ml^{-1} protein in 20 m*M* Tris–HCl buffer pH 7.5 based on the absorbance at 280 nm with an absorption coefficient of 0.88 mg^{-1} ml cm^{-1}. We checked that this concentration is appropriate for crystallization screening using Pre-Crystallization Test (PCT, Hampton Research). The initial crystallization screening was carried out by the sitting-drop vapour-diffusion technique with a 96-well crystallization screening plate (Corning) at 293 K using the commercially available sparse-matrix screening kits Crystal Screen, Crystal Screen II (Hampton Research) and Wizard I and II (Emerald BioSystems). Each sitting drop was prepared by mixing 0.8 µl sample solution and 0.8 µl reservoir solution and was equilibrated against 100 µl reservoir solution. Subsequent optimization of the initial crystallization condition was performed by the hanging-drop vapour-diffusion method using a 24-well VDX plate (Hampton Research) at 293 K, with variation of buffer pH, precipitant concentration and the use of various additives. Each hanging drop was prepared by mixing 1.6 µl sample solution and 0.8–3.2 µl reservoir solution. Each drop was equilibrated against 500 µl reservoir solution.

2.3. X-ray diffraction analysis

Prior to X-ray diffraction experiment, the AldT crystal was flash-cooled at 100 K under a stream of nitrogen gas after briefly soaking in an appropriate crystallization solution supplemented with 20%(*v*/*v*) glycerol. The X-ray diffraction data were collected on a Rigaku R-AXIS IV^{++} imaging-plate area-detector system mounted on a Rigaku MicroMax-007 rotating copper-anode X-ray generator operating at 40 kV and 20 mA. Each oscillation frame was taken with a



(*a*)

(*b*)

(*c*)

Figure 1
Crystals of AldT. (*a*) The initial crystals appeared in a heavy amorphous precipitate under the following conditions: 0.1 *M* sodium acetate pH 4.6, 25%(*w*/*v*) PEG 4000 and 0.2 *M* ammonium sulfate. (*b*) A large grain-shaped crystal was obtained using 0.1 *M* sodium acetate pH 4.6, 18%(*w*/*v*) PEG 4000 and 0.4 *M* ammonium sulfate. This crystal did not diffract beyond a resolution of 4.0 Å. (*c*) A crystal with hexahedral morphology grown in 0.1 *M* sodium acetate pH 4.6, 18%(*w*/*v*) PEG 4000, 0.2 *M* ammonium sulfate and 15%(*v*/*v*) glycerol. This crystal was used for X-ray diffraction data collection to 2.8 Å resolution.

rotation of 1.0° for a 4 min exposure at a crystal-to-detector distance of 200 mm; the total rotation range for the data collection was 180°. The measured diffraction intensities were integrated, scaled and merged with the *HKL*2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

The first crystals of AldT appeared in a heavy amorphous precipitate under several acidic conditions in combination with polyethyleneglycol (PEG) and ammonium sulfate as precipitants. These crystals were grain-shaped with maximum dimensions of approximately 50 µm (Fig. 1*a*). After optimization of the initial conditions by varying pH values and precipitant concentrations, a single crystal with approximate dimensions of 150 × 100 × 100 µm was obtained within a few days using reservoir solution containing 0.1 *M* sodium acetate pH 4.6, 18%(*w*/*v*) PEG 4000 and 0.4 *M* ammonium sulfate (Fig. 1*b*). However, the preliminary X-ray diffraction analysis showed that this crystal did not diffract beyond a resolution of 4.0 Å and the unit-cell parameters could not be determined owing to a high mosaic spread (data not shown). In order to obtain crystals of higher quality, we subsequently used various salts and organic compounds as additives for optimizing the initial conditions. Finally, a different crystal form with a hexahedral morphology was obtained by adding 15–20%(*v*/*v*) glycerol to the original reservoir solution. A single crystal with approximate dimensions of 100 × 100 × 50 µm used for X-ray diffraction data collection was grown within two weeks using a reservoir solution containing 0.1 *M* sodium acetate pH 4.6, 18%(*w*/*v*) PEG 4000, 0.2 *M* ammonium sulfate and 15%(*v*/*v*) glycerol (Fig. 1*c*).

This crystal diffracted to a resolution of 2.8 Å using in-house Cu *Kα* radiation and an almost complete X-ray diffraction data set was collected. The crystal was found to belong to the primitive hexagonal or primitive trigonal system, with unit-cell parameters $a = b = 82.0$, $c = 138.7$ Å. The $R_{\rm sym}$ value calculated in the data-merging process clearly showed that the crystal belongs to the trigonal space group and the reflection condition for 00*l* indicated that the space group of the crystal must be $P3_121$ or its enantiomorph $P3_221$. Assuming the presence of two AldT monomers in the asymmetric unit, the calculated value of the crystal volume per protein weight ($V_{\rm M}$ value; Matthews, 1968) is 2.2 Å^3 Da^{-1}. This value corresponds to a solvent content of 44.4%. The tetrameric structure of AldT can be generated by crystallographic twofold symmetry. The crystallographic parameters and the data-collection statistics are summarized in Table 1.

Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Rotating anode
Wavelength (Å)	1.5418 (Cu *Kα* radiation)
Resolution (Å)	50–2.80 (2.90–2.80)
Unit-cell parameters	
a (Å)	82.0
b (Å)	82.0
c (Å)	138.7
Mosaicity (°)	0.41
Space group	$P3_121$ or $P3_221$
No. of reflections measured	145004
No. of unique reflections measured	13811
$R_{\rm sym}$†	0.140 (0.435)
Data completeness (%)	99.8 (100.0)
Data redundancy	10.5 (10.7)
Average $I/\sigma(I)$	26.1 (8.8)

† $R_{\rm sym} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$, where $\langle I_{\rm h} \rangle$ is the mean intensity of a set of equivalent reflections.

To date, several crystal structures of SDR family proteins have been reported. The *3D-PSSM* server (Kelley *et al.*, 2000) picked out several SDR proteins from the PDB that have a similar structure. Pairwise sequence alignments with *CLUSTALW* (Thompson *et al.*, 1994) between AldT and the enzymes listed by the *3D-PSSM* server suggested that BmGlcDH (Yamamoto *et al.*, 2001) is expected to have highest structural similarity to AldT (31% sequence identity; Fig. 2). Molecular replacement by the program *AMoRe* (Navaza, 1993) and *MOLREP* (Collaborative Computational Project, Number 4, 1994) is in progress using various fragments of the BmGlcDH structure as search probes. In addition, preparation of heavy-atom derivatives for multiple isomorphous replacement is currently being attempted.

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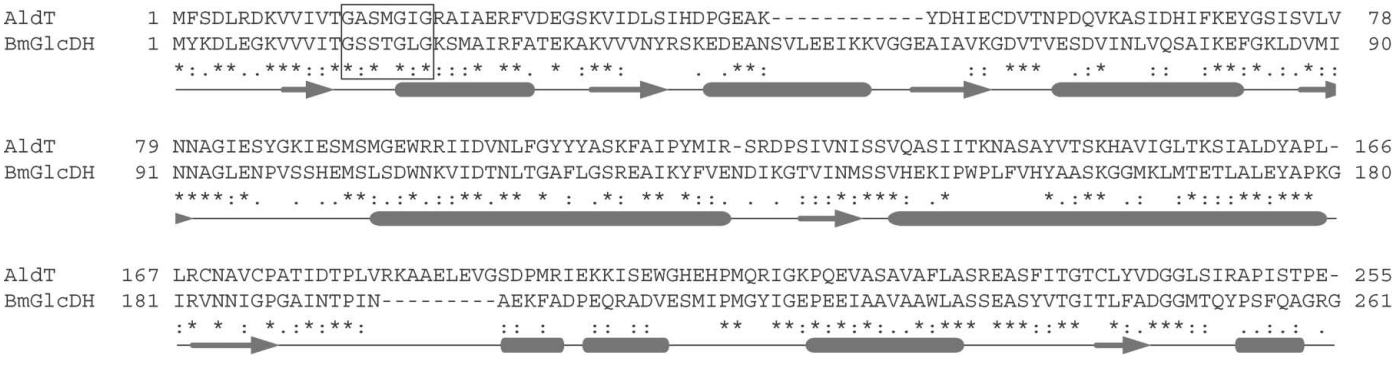

Figure 2
Sequence alignment between AldT and BmGlcDH. The secondary structure of BmGlcDH is also shown. The N-terminal six *β*-strands connected by five *α*-helices represent a typical Rossmann-fold motif responsible for dinucleotide binding. The consensus sequence G*XXXGX* (where *X* is any amino acid) of the Rossmann fold is boxed. Identical, strongly similar and weakly similar residues are denoted by asterisks, semicolons and colons, respectively. The sequence alignment was performed using *CLUSTALW* (Thompson *et al.*, 1994).

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