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Crystallization and preliminary X-ray characterization of an NAD(P)-dependent butanol dehydrogenase A from *Geobacillus thermodenitrificans* NG80-2

Geobacillus thermodenitrificans NG80-2 encodes two long-chain NAD(P)-dependent alcohol dehydrogenases, gtADH1 and gtADH2, in the terminal oxidation pathway of long-chain *n*-alkanes for the conversion of long-chain alkyl alcohols to their corresponding aldehydes. Both gtADH1 and gtADH2 are thermostable enzymes and oxidize long-chain alkyl alcohols up to at least C₃₀. In order to understand the structural basis for their role in long-chain alkane degradation, we have crystallized gtADH2. Single, colourless crystals were obtained from a recombinant preparation of ADH2 overexpressed in *Escherichia coli*. The crystals belong to space group C222₁, with unit-cell parameters *a* = 56.0, *b* = 99.6, *c* = 123.1 Å. Diffraction data were collected in-house to 1.79 Å resolution. The crystals contain one monomer in the asymmetric unit, with a *V*_M value of 2.17 Å³ Da⁻¹ and an estimated solvent content of 43%.

1. Introduction

Alcohol dehydrogenases (ADHs) (EC 1.1.1.1) are a family of oxidoreductases that catalyse the interconversion between alcohols and their corresponding aldehydes or ketones (Ying *et al.*, 2007), and are widely present in all organisms. ADHs exhibit a broad substrate range and play a diverse range of physiological roles (Radianingtyas & Wright, 2003). In alkane biodegradation, ADHs are constituent members of the terminal oxidation pathway (alkane → alkyl alcohol → alkyl aldehyde → fatty acid) and catalyse the second reaction step. NAD(P)-dependent ADHs can be divided into three major groups based on their molecular properties and metal content. Group I includes the Zn-containing 'long-chain' ADHs; group II includes 'short-chain' metal-free ADHs; and group III contains Fe-containing/activated 'long-chain' ADHs (Liu *et al.*, 2009). There has been increasing interest in studying ADHs (Radianingtyas & Wright, 2003), but their importance and the catalytic mechanism of iron in such enzymes are not well understood.

Geobacillus species, literally meaning earth or soil *Bacillus*, are widely distributed and readily isolated from natural and man-made thermophilic biotopes (McMullan *et al.*, 2004). Members of *Geobacillus*, with growth temperatures ranging from 308 to 351 K (Nazina *et al.*, 2001; Feng *et al.*, 2007; Svetlitsnyi *et al.*, 1996; Wang *et al.*, 2006), have attracted considerable interest for their potential industrial applications in biotechnological processes as sources of various thermostable enzymes, such as thermostable proteases (Sookkheo *et al.*, 2000) and lipases (Lee *et al.*, 2001), which are advantageous in certain applications because of their high activities at elevated temperatures and stabilities in organic solvents (Niehaus *et al.*, 1999). *G. thermodenitrificans* NG80-2, a thermophilic bacillus isolated from a deep oil reservoir in northern China, degrades long-chain *n*-alkanes (C₁₅ to C₃₆) via the terminal oxidation pathway between 318 and 346 K (Feng *et al.*, 2007). The terminal oxidation pathway in this bacterium has been well characterized using a proteomics approach (Feng *et al.*, 2007). The first gene in the pathway, *ladA*, encodes a mono-oxygenase that serves as the key initiating enzyme, responsible for converting long-chain alkanes to the corresponding alkyl alcohols (Feng *et al.*, 2007); its three-dimensional structure was reported by Li *et al.* (2008). Two genes encode alcohol

dehydrogenases (*adh1* and *adh2*) for the conversion of alkyl alcohols to the corresponding alkyl aldehydes (Liu *et al.*, 2009). The final gene in the alkane degradation pathway, *aldh*, encodes an aldehyde dehydrogenase for the conversion of alkyl aldehydes to fatty acids. The fatty acids are then degraded to acetyl-CoA via β -oxidation before entering the tricarboxylic acid (TCA) cycle (also known as the citric acid or Krebs cycle) or glyoxylate bypass when alkanes are present as the sole carbon source for energy production (Feng *et al.*, 2007).

G. thermodenitrificans NG80-2 ADH1 and ADH2 (gtADH1 and gtADH2) are both categorized as members of the group III Fe-containing/activated 'long-chain' ADH family (pfam00465) on the basis of their primary sequence. However, gtADH1 and gtADH2 share only 26% sequence identity, suggesting they have different origins. Both gtADH enzymes feature an iron-binding motif (GXXHXXAHXXGXXXXPHG) that has been proposed as a putative fingerprint motif for the group III ADHs, although biochemical analysis showed that iron enhances their activity but is not absolutely required for their activity (Feng *et al.*, 2007), unlike other Fe-activated ADH enzymes. Both gtADH1 and gtADH2 are indispensable for the survival of NG80-2 in oil reservoirs, and both have been shown to be expressed at similar levels in NG80-2 cells supplanted with hexadecane or by sucrose, as confirmed by 2-DE (two-dimensional gel electrophoresis) analysis (Feng *et al.*, 2007). In the case of ADH2, the presence of an excess of alkyl alcohols is required before the oxidation reaction can take place and this situation may occur when alkanes are utilized as carbon and energy sources.

To date, there are no structures available for an NAD(P)-dependent butanol dehydrogenase A that oxidizes long-chain alkyl alcohols. A BLAST search reveals that the most closely related structures to gtADH2 in the Protein Data Bank (PDB) are an Fe-containing NADH-dependent butanol dehydrogenase A (TM0820) from *Thermotoga maritima* at 1.78 Å resolution (44% sequence identity; PDB code 1vlj, Joint Center for Structural Genomics, unpublished work) and the Zn-containing alcohol dehydrogenase YqhD from *Escherichia coli* at 2.0 Å resolution (40% sequence identity; PDB code 1oj7, Sulzenbacher *et al.*, 2004). Given its role in long-chain alkane degradation, gtADH2 has potential applications in crude oil remediation and other bioconversion processes, such as the conversion of long-chain alkyl alcohols and alkyl aldehydes in waxes used in the perfume and flavour industries. In order to explore the structural basis for long-chain alkyl alcohol degradation by alcohol dehydrogenases, here we report the expression, purification and preliminary X-ray crystallographic analysis of recombinant gtADH2.

2. Materials and methods

2.1. Protein expression and purification

The expression and purification of gtADH2 were performed as described by Liu *et al.* (2009) with some modifications. Briefly, the *GTNG_2878* gene encoding gtADH2 (GenBank accession number: ABO68223.1) was cloned from *G. thermodenitrificans* NG80-2 and subcloned into a pET-28a(+) vector (Novagen) with a hexahistidine tag at the N-terminus. *E. coli* strain BL21 (DE3) (Novagen) carrying the recombinant plasmid was grown in 1 l of Luria–Bertani broth (LB) medium containing 100 mg l⁻¹ kanamycin to an OD₆₀₀ of 0.6 and the expression of gtADH2 was induced by 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 318 K for a further 2.5 h. The cells were harvested by centrifugation at 5000 rev min⁻¹ for 15 min, resuspended in lysis buffer consisting of 50 mM Tris–HCl pH 8.0,

Table 1

Data-collection statistics for ADH2.

Values in parentheses are for the highest-resolution shell.

Unit-cell parameters (Å)	$a = 56.0, b = 99.6, c = 123.1$
Space group	C222 ₁
Wavelength (Å)	1.5418
Resolution range (Å)	50–1.79 (1.82–1.79)
Measured reflections	230659
Unique reflections	33677
Completeness (%)	99.2 (89.5)
Redundancy	6.5 (5.3)
$\langle I/\sigma(I) \rangle$	40.3 (2.0)
$R_{\text{merge}}^{\dagger}$ (%)	4.4 (82.1)

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $\langle I(hkl) \rangle$ is the mean intensity of the observations $I_i(hkl)$ of reflection hkl .

300 mM NaCl, 10 mM imidazole and disrupted by sonication. Protein purification was carried out at 277 K using the following procedures. The supernatant obtained by centrifugation at 18 000g for 40 min was applied onto a nickel-affinity chromatography column (2.0 ml of Ni²⁺–NTA agarose) pre-equilibrated with lysis buffer. Unbound proteins were washed out with wash buffer consisting of 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 20 mM imidazole. His-tagged fusion proteins were eluted with elution buffer consisting of 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 250 mM imidazole for about 20 ml. Resource Q anion-exchange chromatography (GE Healthcare, USA) was subsequently applied via NaCl 0–1 M gradient in 50 mM Tris–HCl pH 8.0 buffer. The target protein was finally eluted with approximately 0.05 M NaCl. The ADH was further purified by a Superdex-200 gel-filtration chromatography column (GE Healthcare, USA) in 50 mM Tris–HCl pH 8.0 and 300 mM NaCl and ultrafiltered to 20 mg ml⁻¹ for subsequent crystallization.

2.2. Crystallization

Initial crystallization trials were carried out at 289 K using the sitting-drop vapour-diffusion method in 48-well plates (XtalQuest) with the commercial screening kits Crystal Screen, Crystal Screen 2 and Index from Hampton Research and Wizard I, II, III and IV from Emerald BioSystems. Each crystallization drop contained 1 μ l protein solution and 1 μ l reservoir solution and was equilibrated against 100 μ l reservoir solution. The most promising crystallization conditions were found to be 100 mM phosphate–citrate, pH 4.2, 20% (w/v) PEG 8000, 200 mM NaCl. These conditions were optimized manually

Figure 1

gtADH2 crystals grown in 100 mM phosphate–citrate, pH 6.0, 12.5% (w/v) PEG 8000, 200 mM NaCl. The approximate size of the crystals is indicated by the scale bar.

Figure 2
Sample X-ray diffraction image. (a) The low- (50 Å) and high-resolution (1.79 Å) limits are indicated by the blue hashed areas. (b) An enlarged view showing spots to the edge of the diffraction pattern.

Figure 3
Size-exclusion chromatograph showing that gtADH2 is a dimer in solution.

to 100 mM phosphate–citrate, pH 6.0, 12.5% (w/v) PEG 8000, 200 mM NaCl with the hanging-drop vapour-diffusion method at 289 K, by mixing 1 µl of protein solution [15 and 20 mg ml⁻¹ protein, respectively, in 20 mM Tris–HCl (pH 8.0) and 10 mM NaCl] and 1 µl reservoir solution.

2.3. Data collection

Crystals were flash-cooled in a liquid-nitrogen stream and stored in liquid nitrogen prior to data collection. The 1.79 Å resolution data set for ADH crystals was collected in-house from a single crystal at 100 K

using a Rigaku R-Axis-HTC detector and a Rigaku MM-007HF X-ray source. The reservoir solution with 20% (v/v) glycerol was used as cryoprotectant and data were collected at 100 K. X-ray diffraction intensity data were indexed, integrated, scaled and merged with the program *HKL-2000* (Otwinowski & Minor, 1997). The space group of the gtADH2 crystals was determined to be *C222*₁. The phases were solved using the molecular-replacement method with the program *Phaser* (McCoy *et al.*, 2007) in the *PHENIX* suite (Adams *et al.*, 2010). Data-collection statistics for gtADH2 are summarized in Table 1.

3. Results and discussion

The *GTNG_2878* gene from *G. thermodenitrificans* NG80-2 was successfully cloned into *E. coli* and the encoded NAD(P)-dependent butanol dehydrogenase A with a hexahistidine tag at the N-terminus was purified to homogeneity by metal-chelating affinity chromatography. The optimal crystallization conditions included 100 mM phosphate–citrate, pH 6.0, 12.5% (w/v) PEG 8000, 200 mM NaCl. Small, cubic-shaped crystals typically appeared in about 1 week (Fig. 1) and diffracted to 1.79 Å resolution in-house (Fig. 2).

The gtADH2 crystals belong to the space group *C222*₁ with unit-cell parameters $a = 56.0$, $b = 99.6$, $c = 123.1$ Å. It is predicted that the crystals contain one monomer in the asymmetric unit, with a V_M value of 2.17 Å³ Da⁻¹ and an estimated solvent content of 43% (Matthews, 1968). However, size-exclusion chromatography suggests that gtADH2 is a dimer in solution (Fig. 3), which is consistent with previous reports that gtADH2 forms a homodimer in solution by MALDI–TOF–MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) (Feng *et al.*, 2007).

Structure determination by molecular replacement is currently in progress using the structure of an NADH-dependent butanol dehydrogenase A (TM0820) from *T. maritima* at 1.78 Å resolution (PDB code 1vlj), which shares 44% identity with *G. thermodenitrificans* ADH2, as a search model. A molecular-replacement solution was found with one molecule per asymmetric unit and, after initial refinement, the working and free *R* factors are 37.0 and 44.2%, respectively. Further refinement of the gtADH2 structure is under way and will be reported elsewhere.

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